Food Research

IN THIS ISSUE

A Photomicrographic Method for Estimating the Quantity and Distribution of Fat in Muscle Tissue

Effect of Xanthophyll on the Palatability and Fat Stability of Fresh and Frozen Broad-Breasted Bronze Turkeys

The Effects of Oven Temperature on Certain Characteristics of Baked Salmon

Enzyme Action and Off-Flavor in Frozen Peas

Effect of Degree of Finish on Differences in Quality Factors of Beef

Effect of Gamma Radiation on Bacterial Spores That Have Undergone Germination Induced by Treatment with L-Amino Acids

Some Factors Influencing Thermal Resistance Values Obtained by the Thermoresistometer Method

Browning of Sugar Solutions. I. Effect of pH and Type of Amino Acid in Dilute Sugar Solutions

Browning of Sugar Solutions. II. Effect of the Position of Amino Group in the Acid Molecule in Dilute Glucose Solutions

A Method for the Characterization of Pectic Substances in Some Fruit and Sugar-Beet Marcs

Effect of Certain Ion Combinations Commonly Found in Potable Water on Rate of Filtration Through Roasted and Ground Coffee

The Effect of Blanching on the Carbonyl Content of the Crude Lipid During the Storage of Frozen Peas

Roasting of Frozen Stuffed Turkeys. I. Survival of *Salmonella Pullorum* in Inoculated Stuffing

Roasting of Frozen Stuffed Turkeys. II. Survival of *Micrococcus Pyogenes* var. *Aureus* in Inoculated Stuffing

Volatile Carbonyl Compounds of Cooked Chicken. I. Compounds Obtained by Air Entrainment

Effect of Non-Antiscorbutic Reducing Substances upon the Ascorbic Acid Content of Baked Potatoes

Optimum Conditions of Hydrolysis for Microbiological Assay of Cystine in Food Proteins

Notes and Letters

Effect of Metabolites on the Viability of Heat-Treated *Pseudomonas Fluorescens*

The Role of Methional as a Flavor Compound

Volume 23, Number 1 ***January-February, 1958***

AN OFFICIAL PUBLICATION OF

THE INSTITUTE OF FOOD TECHNOLOGISTS

Food Research

An Official Publication of

The Institute of Food Technologists

Volume 23 JANUARY-FEBRUARY, 1958 Number 1

EDITORIAL BOARD

MARTIN S. PETERSON, *Editor-in-Chief*
(Editorial Office: 11606 South Bell Avenue,
Chicago 43, Illinois)

FRED E. DEATHERAGE
J. L. ETCHELLS
C. A. GREENLEAF
S. W. F. HANSON
MARGARET IVES
DANIEL MELNICK
R. T. MILNER
J. H. MITCHELL, JR.
HAROLD W. SCHULTZ
CHARLES R. STUMBO
REESE H. VAUGHN
R. O. WAGENAAR

Z. I. KERTESZ, *Consulting Editor*

CHARLES S. LAWRENCE, *Business Manager*
176 West Adams Street
Chicago 3, Illinois

Copyright, 1958
By the Institute of Food Technologists
Printed in U. S. A.

Published by the Institute of Food Technologists six times a year. Printed by The Garrard Press, 510-522 N. Hickory Street, Champaign, Illinois. Entered as second-class matter at the Post Office at Champaign, Illinois, under the act of March 3, 1879. Accepted for mailing at special rate of postage provided for in amended Section 34.40(e), pursuant to the provisions of Public Law 233, approved October 8, 1951.

Food Technology

Published Monthly by the

INSTITUTE OF FOOD TECHNOLOGISTS

The Companion Journal to

FOOD RESEARCH

If you are a reader of FOOD RESEARCH your library is incomplete without FOOD TECHNOLOGY—devoted to the publication of the results of research and their practical application in industry. Contributors to its pages represent many fields of scientific and technological interest.

SUBSCRIPTION RATES FOR 1958

FOOD RESEARCH	$8.50 Domestic; $10.00 Foreign
FOOD TECHNOLOGY . . .	$8.50 Domestic; $10.00 Canada, Cuba, and Mexico; $12.00 all other foreign.

Send Subscriptions to

FOOD TECHNOLOGY

THE GARRARD PRESS

510-522 N. Hickory Street

CHAMPAIGN, ILLINOIS

Volume 23 JANUARY-FEBRUARY, 1958 Number 1

CONTENTS

A Photomicrographic Method for Estimating the Quantity and Distribution of Fat in Muscle Tissue. Burnadine L. Lewis, Dorothy L. Harrison, and Dean S. Folse 1

Effect of Xanthophyll on the Palatability and Fat Stability of Fresh and Frozen Broad-Breasted Bronze Turkeys. Burnadine L. Lewis, Dorothy L. Harrison, and Paul E. Sanford 7

The Effects of Oven Temperature on Certain Characteristics of Baked Salmon. Helen Charley and Grayce E. Goertz 17

Enzyme Action and Off-Flavor in Frozen Peas. A. C. Wagenknecht and Frank A. Lee 25

Effect of Degree of Finish on Differences in Quality Factors of Beef. Marion Simone, Floyd Carroll, and M. T. Clegg 32

Effect of Gamma Radiation on Bacterial Spores That Have Undergone Germination Induced by Treatment with L-Amino Acids. B. Kan, S. A. Goldblith, and B. E. Proctor 41

Some Factors Influencing Thermal Resistance Values Obtained by the Thermoresistometer Method. J. L. Secrist and C. R. Stumbo 51

Browning of Sugar Solutions. I. Effect of pH and Type of Amino Acid in Dilute Sugar Solutions. C. O. Willits, J. C. Underwood, H. G. Lento, Jr., and C. Ricciuti 61

Browning of Sugar Solutions. II. Effect of the Position of Amino Group in the Acid Molecule in Dilute Glucose Solutions. H. G. Lento, Jr., J. C. Underwood, and C. O. Willits 68

A Method for the Characterization of Pectic Substances in Some Fruit and Sugar-Beet Marcs. Mildred Gee, Elizabeth A. McComb, and Rolland M. McCready 72

Effect of Certain Ion Combinations Commonly Found in Potable Water on Rate of Filtration Through Roasted and Ground Coffee. D. G. Gardner 76

The Effect of Blanching on the Carbonyl Content of the Crude Lipid During the Storage of Frozen Peas. Frank A. Lee 85

Roasting of Frozen Stuffed Turkeys. I. Survival of Salmonella Pullorum in Inoculated Stuffing. Raymond E. Rogers and Millard F. Gunderson . . 87

Roasting of Frozen Stuffed Turkeys. II. Survival of Micrococcus Pyogenes var. Aureus in Inoculated Stuffing. Raymond E. Rogers and Millard F. Gunderson 96

Volatile Carbonyl Compounds of Cooked Chicken. I. Compounds Obtained by Air Entrainment. E. L. Pippen, M. Nonaka, F. T. Jones, and Fred Stitt . . 103

Effect of Non-Antiscorbutic Reducing Substances upon the Ascorbic Acid Content of Baked Potatoes. Janet Wilson McAfee and Johnnie H. Watts . 114

Optimum Conditions of Hydrolysis for Microbiological Assay of Cystine in Food Proteins. S. C. Balasubramanian and M. Ramachandran 119

Notes and Letters

Effect of Metabolites on the Viability of Heat-Treated Pseudomonas Fluorescens. C. D. Heather and C. Vanderzant 126

The Role of Methional as a Flavor Compound. E. A. Day, Mark Keeney, and William H. Stahl 130

SUBSCRIPTION RATES

To members of the Institute of Food Technologists, $7.50 per volume.

Non-members: Domestic, $8.50 per volume; foreign, $10.00 per volume.

Subscriptions and renewals are entered to begin with the first issue of the current volume.

Single copies of current volume: Domestic, $1.50; foreign, $1.75.

CORRESPONDENCE

Editorial: Martin S. Peterson, Editor-in-Chief, 11606 South Bell Ave., Chicago 43, Ill.

Non-Member Subscriptions, Back Volumes, Single Copies, and Reprints: Food Research, 510-522 N. Hickory Street, Champaign, Ill.

Member Subscriptions: The Institute of Food Technologists, 176 W. Adams St., Chicago 3, Ill.

Advertising: Charles S. Lawrence, Business Manager, 176 W. Adams St., Chicago, Ill.

CLAIMS

Claims for copies lost in the mails must be received within 30 days (90 days foreign) of the date of issue. Notice of change of address must be received two weeks before date of issue.

NOTE

The Institute of Food Technologists assumes no responsibility for statements in articles appearing in Food Research. The opinions expressed do not necessarily reflect policies of the Institute of Food Technologists.

Manuscripts for Food Research should conform to the style used in the journal and be submitted in duplicate to the Editor-in-Chief. Authors of papers intended for Food Research should request from the Editorial office a copy of the booklet "Information and suggestions for authors." Food Research reserves the privilege of editing manuscripts to make them conform with the adopted style of the journal or returning them to the author for revision. Changes made by the Editorial Office may be reviewed by authors when they examine proof.

An Authorized Binding

for

FOOD RESEARCH

Arrangements have been made by Institute of Food Technologists for subscribers to have their journals bound into distinctively designed books.

Six issues, January through December, bound in best grade brown washable buckram, imprinted with your name on cover, cost but $3.95 per volume.

Bound journals serve as an immediate reference for research and information. Properly displayed, they create a psychological impact, implying the time and effort spent to keep up-to-date on the most modern methods and materials.

Ship journals parcel post. Within thirty days after receipt, bound volumes will be shipped prepaid anywhere in the U. S. A. Full remittance must accompany order.

* * *

Publishers' Authorized Bindery Service

(Binders of all Journals)

5811 W. Division St. **Chicago 51, Illinois**

A PHOTOMICROGRAPHIC METHOD FOR ESTIMATING THE QUANTITY AND DISTRIBUTION OF FAT IN MUSCLE TISSUE[a,b]

BURNADINE L. LEWIS[c], DOROTHY L. HARRISON, AND DEAN S. FOLSE

Departments of Foods and Nutrition and Pathology, Kansas State College, Manhattan, Kansas

(Manuscript received July 21, 1956)

Flavor, juiciness, and tenderness are probably the most important properties that contribute to the palatability of muscles from animals used for meat. It is agreed that the amount, distribution, and composition of the fat affect the flavor of both fresh and frozen stored poultry. Lowe *(2)* listed the finish and distribution of fat in poultry as factors which may influence its juiciness. Furthermore, muscle tissue well marbled with fat is associated with tenderness. An objective method for estimating the quantity and distribution of fat in the histological study of muscle tissue would be of great usefulness.

Wang et al. (3) asserted that it is not the total amount of fat in a muscle, but the way it is distributed throughout the muscle that affects tenderness appreciably. They measured the "linear fat," i.e., the longest axis of each fat island in raw microscopic sections of beef, and consistently found that the amount of "linear fat" in the sections of raw meat correlated well with the tenderness scores for the cooked samples.

Although there are chemical methods for determining fat quantitatively, a method that provides information regarding fat distribution as well as quantity in muscle has apparently not been devised. Therefore, the study reported herein was initiated to: (a) devise a photomicrographic method for estimating the quantity and distribution of fat in muscle tissue; and (b) compare the photomicrographic method with a numerical scoring method adapted from the method reported by Ramsbottom, Strandine, and Koonz *(2)* for estimating the quantity of connective tissue in muscle.

MATERIALS AND METHODS

Raw and cooked tissue from the *pectoralis major* and *gluteus primus* muscles of Broad-Breasted Bronze turkeys, fed a control ration and a control ration supplemented with xanthophyll, were used in this study. Small blocks of tissue, preserved in physiological salt and formalin solution, were frozen on a clinical microtome, and longitudinal sections 15 to 25 μ thick were cut.

The sections were stained with Herxheimer's scarlet red and Harris' hematoxylin, and mounted in glycerine jelly. Herxheimer's scarlet red imparted a bright red-orange to the fat, whereas Harris' hematoxylin stained the muscle fibers bluish-purple. The connective tissue was a darker bluish-purple to grey.

---

[a] Contribution No. 192, Department of Home Economics, and No. 147, Department of Veterinary Medicine, Kansas Agricultural Experiment Station, Manhattan.

[b] The data are from a thesis presented to the Graduate Faculty by Burnadine L. Lewis in partial fulfillment of the requirements for the degree Doctor of Philosophy.

[c] Present address: Prairie View, Texas.

A total of 1600 slides was prepared from 320 blocks of tissue; 160 raw and 160 cooked samples were equally distributed between the 2 rations and the 2 muscles. Two sections were mounted on each of almost all slides.

Numerical scoring method. The relative quantity of intramuscular fat was estimated by assigning a numerical score to each section. A score of 7, 5, 3, and 1 represented a large, medium, and small quantity of fat, and no fat, respectively. All the sections prepared were scored in this manner, and an average score was calculated for each muscle from each bird.

Photomicrographic method. Both the quantity and the distribution of fat in the muscles were estimated by a photomicrographic method. For this part of the study, 100 sections of raw tissue were selected. These sections, 50 from the *pectoralis major* and 50 from the *gluteus primus* muscle were considered representative of, and were equally divided among, tissues from birds on the 2 rations.

Commercial photomicrographic equipment was set up with the camera and microscope in a vertical position. Paper negatives (made by exposing contact paper) were made of sections prepared from muscle tissue. An ocular micrometer placed in the eyepiece of the microscope produced a pattern on the paper negatives at the same time that the exposure of the section of tissue was made (Figure 1). A 5 X ocular, a 47.5 X objective, and a bellows extension of 20 inches gave a magnification of 475 X.

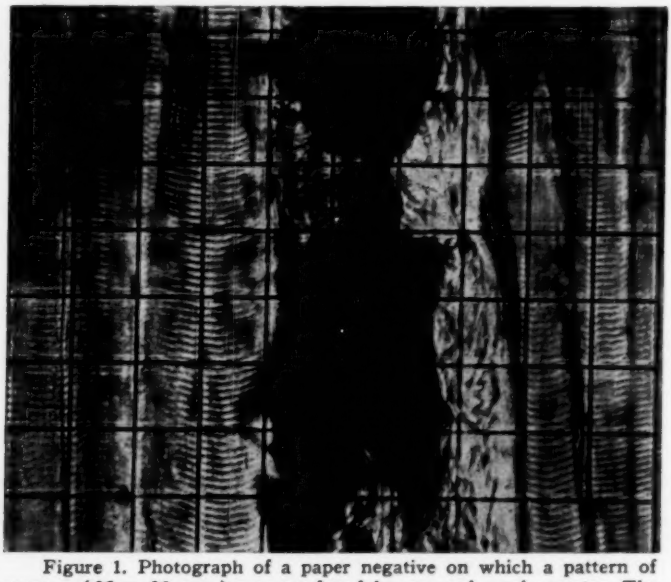


Figure 1. Photograph of a paper negative on which a pattern of squares (.06 x .06 mm.) was produced by an ocular micrometer. The dark area in the center of the photograph is fat.

The size of the squares made by the ocular micrometer was determined by making a negative of a stage micrometer set at the magnification given above. The stage micrometer produced a pattern on the negative which, when superimposed on the paper negatives, showed that the area of the squares produced by the ocular micrometer was 0.0036 square millimeter.

A negative was made of a hemocytometer for the purpose of producing a pattern of squares of smaller size than those produced by the ocular micrometer. The large squares in the center of the hemocytometer are divided into 16 squares of equal size.

A 5 X ocular, a 10 X objective and a bellows extension of 12.25 inches used to photograph the hemocytometer produced on the negative a pattern of large squares of the same dimension as those made on the paper negatives by the ocular micrometer. The area of the small squares on the negative of the hemocytometer was calculated to be 0.000225 sq. mm.

By superimposing the negative of the hemocytometer on the paper negatives of muscle tissue, the number of small squares filled with fat could be determined. The number of squares times the area of each square gave an estimate of the quantity of fat in a total area of 0.09 sq. mm. The number of large squares in which fat appeared (without regard to the quantity therein) was used as an indication of fat distribution.

RESULTS AND DISCUSSION

Numerical scoring estimate. A summary of the scores for the relative quantity of fat in sections of raw and cooked turkey muscle is given in Table 1. The scores suggest that there was no difference in the quantity of fat observed in sections of muscle from birds fed xanthophyll and birds on a control ration. Some difference in the quantity of fat was observed in the 2 muscles studied. It was estimated that the sections from the *pectoralis major* muscle contained a medium quantity of fat, whereas those from the *gluteus primus* contained a medium to large quantity, Table 1. The slightly larger estimated quantity of fat in the *gluteus primus* than in the *pectoralis major* agrees with data reported by Goertz, Vail, Harrison, and Sanford (1), who made similar observations.

TABLE 1

Average scores[1] for the relative quantity of fat in sections from raw and cooked turkey muscle.

Muscle	Ration	
	Xanthophyll	Control
Pectoralis major		
Raw	4.5	4.3
Cooked	4.8	5.2
Average	4.7	4.8
Gluteus primus		
Raw	6.6	6.0
Cooked	6.2	5.7
Average	6.4	5.9

[1] 7—large quantity
5—medium quantity
3—small quantity
1—no fat

Photomicrographic estimate. Average values for the estimated quantity and distribution of fat in sections of raw turkey muscle as determined by the photomicrographic method are presented in Table 2. These data also indicate no difference in the calculated quantity of fat in sections from birds fed xanthophyll and those on a control ration. This was substantiated by analysis of variance of the combined values for the 2 muscles, Table 3. There appeared to be a slightly greater calculated quantity of fat in the *gluteus primus* than in the *pectoralis major* muscle. This also was suggested by the numerical scores.

The distribution of fat was somewhat different in sections from the *pecto-*

TABLE 2

Average values obtained by the photomicrographic method for the quantity and distribution of fat in sections of raw turkey muscle

Factor	Muscle	Ration	
		Xanthophyll	Control
Quantity, area/.09 sq. mm.	Pectoralis major	0.040	0.044
	Gluteus primus	0.050	0.057
	Difference	0.010	0.013
Distribution, % of .09 sq. mm. areas showing fat	Pectoralis major	63.3	71.1
	Gluteus primus	81.1	81.1
	Difference	17.8	10.0

TABLE 3

F values for the estimated quantity and distribution of fat in sections of raw turkey muscle

Factor	Xanthophyll vs. Control	Pectoralis major vs. gluteus primus
Quantity	0.54 ns	2.52 ns
Distribution	0.40 ns	4.13 ns

ns—nonsignificant

ralis major muscle from that in sections from the *gluteus primus*. On the whole, the fat in the *pectoralis major* sections occurred in large clusters. Usually one or two clusters occurred in each section, and they tended to push the muscle fibers to one side. There was little fat, if any, in other areas of the sections from the *pectoralis major*, Fig. 2. Large and small groups of cells, well distributed throughout the entire section, were characteristic of the fat distribution in the *gluteus primus* muscle. The fat cells in this muscle usually were lined up in narrow rows between the muscle fibers. These rows of fat cells continued from one end of the section to the other, and there were several rows in each section. The same pattern of fat distribution in these muscles from turkey was described by Goertz, Vail, Harrison, and Sanford *(1)*.

The photomicrographic method indicated, on the average, that fat was distributed over a larger area (approximately 14%) in the *gluteus primus* than in the *pectoralis major* sections, Table 2. However, analysis of variance pointed out that differences between the values for areas showing fat in sections from the 2 muscles were nonsignificant.

Similar results were obtained by estimating the quantity of fat with the numerical scoring and photomicrographic methods. However, it is believed

Figure 2. Distribution of fat in the *pectoralis major* muscles. The large cluster of fat cells in the center of the photomicrograph have pushed the muscle fibres above the cluster out of alignment. Usually one or two such clusters of fat occurred in each section from the *pectoralis major*.

that the photomicrographic method has certain advantages. When the photomicrographic method is used, previous determination of the number of sections from a given population that should be examined to obtain valid data will reduce the number of slides required for study. The distribution of fat in tissue can be studied simultaneously with the estimation of the quantity of fat. Also, the

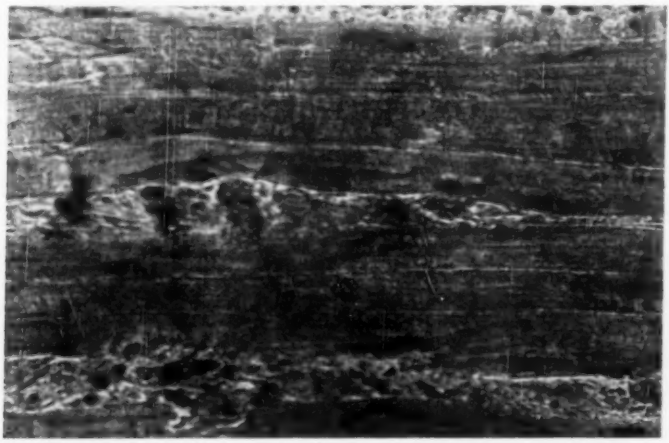


Figure 3. Distribution of fat in the *gluteus primus* muscle. Many small groups of cells were well distributed throughout the section by forming continuous narrow rows of fat from one end of the section to the other.

photomicrographic method tends to eliminate human error encountered when an observer assigns an arbitrary score to an estimated quantity of fat in a section of muscle tissue.

SUMMARY

A method employing photomicrography was developed for estimating the quantity and distribution of fat in muscle tissue. An estimate of the quantity of fat in two muscles from Broad Breasted Bronze turkeys using this method was compared with results obtained when the fat in the tissues from these muscles was estimated by assigning numerical scores. Similar results were obtained by the two methods. However, the photomicrographic is more objective than the numerical scoring method and requires fewer slides than does the numerical scoring method. The distribution, as well as the quantity, of fat in the tissue may be determined by the photomicrographic method.

Acknowledgment

The authors are indebted to the late Dr. W. M. McLeod, formerly Head of the Department of Anatomy, for the use of the photomicrographic equipment and for assistance in taking and developing the photomicrographs.

LITERATURE CITED

1. GOERTZ, G. E., VAIL, G. E., HARRISON, D. L., AND SANFORD, P. E. Organoleptic and histological characteristics of fresh and frozen stored turkey fed high-density and low-density rations. *Poultry Sci.*, **34**, 640 (1955).
2. LOWE, B. Factors affecting the flavor of poultry with emphasis on histological post mortem changes. *Advances in Food Research*, **1**, 203 (1948).
3. RAMSBOTTOM, J. M., STRANDINE, E. J., AND KOONZ, C. H. Comparative tenderness of representative beef muscles. *Food Research*, **10**, 497 (1945).
4. WANG, HSI, RASCH, E., BATES, V., BEARD, F. J., PIERCE, J. D., AND HANKINS, O. G. Histological observations on fat loci and distribution in cooked beef. *Food Research*, **19**, 314 (1954).

EFFECT OF XANTHOPHYLL ON THE PALATABILITY AND FAT STABILITY OF FRESH AND FROZEN BROAD-BREASTED BRONZE TURKEYS[a,b]

BURNADINE L. LEWIS, DOROTHY L. HARRISON, AND PAUL E. SANFORD

Departments of Foods and Nutrition and Poultry Husbandry, Kansas State College, Manhattan, Kansas

(Manuscript received July 21, 1956)

The problem of fat rancidity in frozen stored poultry is of economic importance. Several attempts have been made to retard rancidity of poultry fat by including certain natural antioxidants or other metabolites in the ration. However, the results of such experiments have not been consistent. Criddle and Morgan *(1)*, Kummerow, Vail, Conrad, and Avery *(7)*, and Kummerow, Hite, and Kloxin *(8)* found that peroxide numbers of the fat from frozen stored turkeys were lowered by the addition of tocopherol to the ration, but tocopherol had little effect on the induction period of the fat or on the organoleptic quality of the meat. Also, Kummerow, Vail, Conrad, and Avery *(7)*, Kummerow, Hite, and Kloxin *(8)*, Hite, Kloxin, Kummerow, Vail and Avery *(4)*, and Hite, Kloxin, and Kummerow *(5)* reported that choline and ethanolamine increased the induction period, but had no significant effect on the peroxide numbers of turkey fat or on organoleptic qualities of the meat. Moreover, Klose, Kummerow, Goertz, and Hanson *(6)* stated that the incorporation of ethanolamine, tocopherol, pyrodoxin, calcium pantothenate, mono-ethanol-ammonium gallate, and betaine in the diets of Broad-Breasted Bronze turkeys for 20 days prior to slaughter did not improve the frozen storage stability of the fat. Likewise, Schreiber, Vail, Conrad, and Payne *(10)* were unable to produce a definite improvement in the stability of poultry fat by the addition of ascorbic acid to the ration.

Mecchi, Pool, and Klose *(9)* found that when chickens and turkeys were fed a basal diet supplemented with 0.10% alphatocopheryl acetate, the stability of the depot fat was increased two- to three-fold for the chicken fat, and four- to six-fold for the turkey fat when compared with fat from control birds.

The failure of the inclusion of these antioxidants or metabolites in the ration to give conclusive evidence for improvement of the stabilization of turkey fat has led to a continuation of research in this area. Xanthophyll has been shown to functon physiologically like the tocopherols. It is possible, therefore, that xanthophyll may exhibit antioxidant properties. Accordingly, the present study was designed to determine the effect of xanthophyll, in the ration of immature turkeys, on the palatability and fat stability of frozen turkey halves. Histological studies were made to determine the effect of xanthophyll, if any, on the characteristics of and the amount and distribution of fat in the tissue from two turkey muscles.

---

[a] Contribution No. 191, Department of Home Economics and No. 222, Department of Poultry Husbandry, Kansas Agricultural Experiment Station, Manhattan.

[b] The data are from a thesis presented to the Graduate Faculty by Burnadine L. Lewis in partial fulfillment of the requirements for the degree Doctor of Philosophy.

MATERIALS AND METHODS

Turkey rations. Broad-Breasted Bronze turkey hens reared in 1952 and 1953 were fed a standard starter ration until the poults were 3 weeks of age, a 28% colorless turkey starter between 3 and 12 weeks, and a 24% colorless turkey grower from 12 to 17 weeks of age. In the first experiment (1952) all turkeys were fed a 20% colorless turkey grower from 17 to 20 weeks of age. At the end of 20 weeks the turkeys were divided into 2 groups. The control group continued to receive a colorless ration which contained 16% of protein. The experimental group received the same ration supplemented with 1% xanthophyll concentrate for the last 6 of the remaining 8 weeks. The guaranteed analysis of the xanthophyll concentrate was: 300 International chick units of vitamin D per gram, 1500 U.S.P. (XIV) units of vitamin A per gram, and 136 International units of vitamin E per pound.

In 1953 the turkeys were divided into 2 groups at the end of 16 weeks. The control group was treated the same as the 1952 control group for the remaining 12 weeks. The experimental group received the same basal ration as that of the 1952 experimental group plus 1% xanthophyll concentrate from 16 weeks of age to the end of the feeding period. In both experiments the birds were slaughtered after 28 weeks.

Killing and dressing. In 1952, 10 hens from each group of birds were debrained, then bled by the "inside stick method." The birds were given a slack scald, 124° F. (51.1° C.), for 30 seconds, and were partially picked by a "rotary drum type" automatic picker. After this treatment the remaining feathers and pin feathers were removed by hand. After singeing and washing the birds were chilled for 12 hours at 34° F. (1.1° C.), then eviscerated and washed again. In 1953 the turkeys were killed and eviscerated at a local commercial locker plant.

Preparation for storage. The birds were cut in half lengthwise with the breast bone left on the right side (turkey back up and head away from the cutter). In 1952, 7 birds were cut in half when fresh, and the remaining 13 were cut in half after 4 months of storage. In 1953 all birds were cut in half when fresh.

Both years, 4 half birds from each group were wrapped loosely in aluminum foil and placed in a refrigerator maintained at 38° F. (3.3° C.) until cooked. The turkey halves to be stored were wrapped in foil, sharp frozen, and held at 0° F. (–17.8° C.) at a commercial locker plant.

Cooking tests. The half turkeys were cooked when fresh, i.e., without freezing, and at the end of 4, 8, 12, and 16 months of frozen storage. The 8 halves which were evaluated fresh were roasted on the second and third day following the time of killing. At the end of each storage period 8 half turkeys were defrosted in a refrigerator at 38° F. (3.3° C.) for 48 hours before roasting. These samples were randomized so that both rations were represented an equal number of times at each cooking period.

The half birds were cooked on racks (cut side down) in individual pans. A piece of aluminum foil was placed under the cut side to prevent drying. Birds were roasted at 300° F. (148.9° C.) either to an internal temperature of 185° F. (85° C.) with the thermometer placed in the thigh or until they had remained in the oven 26 minutes per pound, whichever was the longer cooking period. Volatile, dripping, and total cooking losses were determined for each half bird.

Palatability tests. A palatability committee of 8 members scored the *pectoralis major* (light meat) and *gluteus primus* (dark meat) muscles from the breast and thigh, respectively, for flavor, juiciness, and tenderness, and the meat remaining on the turkey carcasses for aroma. Each judge was given a sample from the same position in every *pectoralis major* and *gluteus primus* muscle. Scores for each factor ranged from 10 to 1 with 10 representing the highest possible score and 1 the lowest. Tenderness scores were based on the number of chews required to completely masticate a piece of turkey of standard size.

Objective tests. Shear values and press fluid yields were measured by the Warner-Bratzler shearing apparatus and the Carver laboratory press, respectively. One-inch cores from the cooked *pectoralis major* muscle were used for the shearing tests, and two 25 g. samples of the cooked ground muscle were used for press fluid determinations.

The fat in the drippings from each half turkey was analyzed for free fatty acids and peroxide oxygen. The drippings were held at –20° F. (–28.9° C.) from 1 to 2 weeks

before analyzing; then they were defrosted at room temperature and placed in the freezer just long enough for the top layer of fat to solidify.

The method for determining peroxide oxygen was a modification of that described by Watts and Peng *(11)*. Ethylene dichloride was the extraction solvent and the extraction time was extended to 3 minutes as suggested by Watt, Hall, Mackintosh, and Vail *(10)*. An aliquot of the ethylene dichloride extract was used to titrate free fatty acids in neutral alcohol.

Statistical analyses. The data for the 2 experiments (1952 and 1953) were analyzed separately, because the turkeys reared in 1952 received xanthophyll for a shorter time than those reared in 1953. Scores for flavor, juiciness, and tenderness of the turkeys were analyzed as follows:

Sources of Variation	*D/F*
Ration	1
Storage period	4
Muscle	1
Ration x storage	4
Ration x muscles	1
Storage x muscles	4
Remainder	64
Total	79

Scores for the *pectoralis major* (light meat) and *gluteus primus* (dark meat) muscles were pooled to study the effect of feeding xanthophyll on the flavor, juiciness, and tenderness of the turkey. Scores for each muscle, but without regard for the ration fed, were used for comparisons of the light and dark meat. When there were significant differences in the main effects and interactions between ration and storage time, ration and muscles, or storage time and muscles, two-way tables of means were analyzed by least significant differences.

Other criteria for measuring the quality of the turkey were: aroma scores, acid numbers and peroxide values for fat in the drippings from roasted half-birds, percentage total cooking losses, and press fluid yields and shear values for the *pectoralis major* muscle. The analysis for these factors was:

Sources of Variation	*D/F*
Ration	1
Storage period	4
Ration x storage	4
Turkeys	30
Total	39

RESULTS AND DISCUSSION

Palatability tests. *Flavor.* Mean scores for flavor, juiciness, and tenderness of birds fed xanthophyll and those on the control ration as well as flavor scores for the *pectoralis major* (light meat) and *gluteus primus* (dark meat) muscles are given in Table I. An array of the mean scores for the 1952 birds and calculation of least significant differences showed that at every storage period turkeys fed xanthophyll had slightly higher flavor scores than those on the control ration, but only at the 12-month storage period was the observed difference statistically significant, Table 1. In 1953 the xanthophyll birds scored significantly ($P<.05$) higher after 4 and 12 months of storage. At 0 and 16 months of storage the control birds rated higher than the xanthophyll birds, but the differences were not significant, Table 1. The small differences and the variability in the average

flavor scores indicate that the inclusion of xanthophyll concentrate in the ration had little effect on the flavor of the turkey meat that was of practical importance. In both experiments flavor scores for the *pectoralis major* were significantly ($P<.05$) higher than those for the *gluteus primus*. The difference in the flavor of these two muscles increased with 12 or more months of storage, Table 1.

TABLE 1

Mean flavor, juiciness, and tenderness scores. Maximum score possible, 10

Factor	Year	Variable	Storage time in months				
			0	4	8	12	16
Flavor	1952	Xanth.	8.5 *	8.1 ns	7.8 ns	7.9 *	6.8
		Cont.	ns	ns	ns	*	ns
			8.5 *	8.0 *	7.5 ns	7.4 *	6.6
		lsd	*=0.34				
	1953	Xanth.	8.3 *	7.8 ns	8.0 *	6.5 ns	6.5
		Cont.	ns	*	ns	*	ns
			8.4 *	7.3 ns	7.7 *	6.1 *	6.6
		lsd	*=0.38				
	1952	PM	8.8 *	8.3 *	7.9 ns	8.0 *	7.6
			*	*	*	*	*
		GP	8.3 *	7.6 *	7.4 ns	7.2 *	5.8
		lsd	*=0.34				
	1953	PM	8.6 *	7.7 *	8.1 *	6.6 *	7.3
				ns	*	*	*
		GP	*	7.5 ns	7.5 *	5.9 ns	5.7
			8.1 *				
		lsd	*=0.38				
Juiciness	1952	Xanth.	7.5 *	7.1 ns	7.3 *	6.9 *	5.9
			ns	ns	ns	*	ns
		Cont.	7.7 *	7.3 ns	7.1 *	6.6 *	5.8
		lsd	*=0.24				
	1953	Xanth.	7.4 *	6.6 ns	6.8 ns	7.1 *	6.3
			ns	ns	*	*	ns
		Cont.	7.6 *	6.5 ns	6.3 ns	6.6 *	6.1
		lsd	*=0.34				
	1952	PM	7.8 *	7.4 ns	7.7 *	6.8 *	6.1
			*	*	*	ns	*
		GP	7.4 *	7.0 ns	7.0 ns	6.7 *	5.6
		lsd	*=0.39				
	1953	PM	7.5 *	6.5 ns	6.8 *	7.4 *	6.6
			ns	ns	*	*	*
		GP	7.4 *	6.6 ns	6.4 ns	6.3 *	5.7

Table 1 Concluded

Factor	Year	Variable	Storage time in months								
			0		4		8		12		16
		lsd	* = 0.34								
Tenderness	1952	Xanth.	8.9	*	8.5	ns	8.8	*	8.3	ns	8.5
			ns		ns		ns		ns		ns
		Cont.	9.2	*	8.6	ns	8.6	ns	8.3	ns	8.4
		lsd	* = 0.36								
	1953	Xanth.	8.8	ns	8.5	ns	8.6	ns	8.4	ns	8.2
			*		ns		ns		*		ns
		Cont.	8.3	ns	8.3	*	8.7	*	8.0	ns	8.1
		lsd	* = 0.32								
	1952	PM	9.3	*	8.9	ns	8.5	ns	8.2	ns	8.5
			*		ns		*		ns		ns
		GP	8.8	ns	8.9	*	9.4	*	8.4	ns	8.3
		lsd	* = 0.36								
	1953	PM	8.5	*	8.2	*	8.5	*	8.0	*	8.3
			ns		*		ns		*		ns
		GP	8.5	ns	8.7	ns	8.8	*	8.4	*	8.0
		lsd	* = 0.32								

Legend:
Xanth.—Xanthophyll
Cont.—Control
PM—*Pectoralis major* muscle (light meat)
GP—*Gluteus primus* muscle (dark meat)
* Significant at the 5% level.

Juiciness. In general, the juiciness scores were about the same for xanthophyll as for control birds. The decline in juiciness scores during storage was not linear. However, in both experiments the significant decrease in average juiciness scores occurred at the same time for xanthophyll and control birds, Table 1. Mean juiciness scores for the *pectoralis major* muscle were slightly higher than those for the *gluteus primus*, except after 4 months of storage in 1953, Table 1. The observed differences were significant ($P<.05$) in 1952, but non-significant in 1953. Even though these differences for 1952 birds were statistically significant they probably are not of practical importance.

Tenderness. The feeding of xanthophyll concentrate did not improve the tenderness of the turkey meat. Similarly to the other palatability factors, tenderness scores were much the same for meat from the xanthophyll and control birds, Table 1. Tenderness scores for the *pectoralis major* and *gluteus primus* indicated that the palatability committee found little consistent difference in the tenderness of these muscles, Table 1. In 1952 the fresh meat of the *pectoralis major* was scored significantly ($P<.05$) higher than the *gluteus primus*, but after 8 months of storage the opposite was true. At all other storage periods differences in the tenderness of the two muscles were non-significant, Table 1.

In 1953 the *gluteus primus* was significantly ($P<.05$) more tender than the *pectoralis major* after 4 and 12 months of storage, whereas the *pectoralis major* was the more tender after 16 months. No significant differences between the two muscles were observed at 0 and 8 months of storage, Table 1.

Aroma. In both 1952 and 1953 analyses of variance of the scores for individual birds showed no significant differences in aroma that were attributable to the ration. Mean aroma scores are given in Table 2. These scores for birds reared in 1953 decreased slightly more during storage than those for birds reared in 1952. For the 1953 birds fed xanthophyll the significant ($P<.05$) decreases in aroma occurred between 0 and 4, 8 and 12, and 12 and 16 months. Aroma scores for 1953 birds on the control ration decreased significantly

TABLE 2

Mean aroma scores[1] for and acid numbers and peroxide values of fat in the drippings from roasted turkey halves

Factor	Storage time in months	Xanthophyll		Control	
		1952	1953	1952	1953
Aroma	0	8.2	8.6	8.4	8.2
		ns	*	ns	*
	4	8.0	7.4	8.4	7.6
		ns	ns	*	ns
	8	7.9	7.9	7.6	7.4
		ns	*	ns	*
	12	7.7	6.9	7.4	6.6
		*	*	*	ns
	16	6.7	6.1	6.6	6.2
	lsd	*0.49	0.74	0.49	0.74
Acid Number[3]	0	1.376	1.555	1.716	1.782
	4	1.292	2.055	1.882	2.178
	8	1.938	1.290	2.064	1.392
	12	1.968	1.638	1.668	1.530
		*	ns	*	ns
	16	7.112	1.268	4.689	1.492
	lsd	*1.54		1.54	
Peroxide Value[4]	0	52.60	76.73	47.88	81.85
		*	ns	*	ns
	4	95.38	90.21	93.20	82.89
	8	112.54	96.27	95.87	95.16
	12	97.64	107.36	92.34	104.95
		*	ns	*	ns
	16	115.61	92.95	116.90	110.18
	lsd	*21.04		21.04	

[1] Maximum score possible, 10.
[3] Expressed as mg. KOH/g. of fat.
[4] Expressed as ml. of N/2000 sodium thiosulfate/g. of fat.
* Significant at the 5% level.
ns—non significant.

($P<.05$) between 0 and 4 and 8 and 12 months of frozen storage. In 1952 the scores for xanthophyll birds did not decrease significantly ($P<.05$) until after 12 months of storage, whereas those for birds on the control ration decreased significantly ($P<.05$) between 4 and 8 months, and again between 12 and 16 months of storage.

Chemical tests. *Acid numbers and peroxide values.* Acid numbers and peroxide values were determined for the fat in the drippings from roasted half-turkeys. These data are presented in Table 2. There were no differences in acid numbers that could be accounted for by the inclusion of xanthophyll in the ration. On the average, the acid numbers for the fat in the drippings from birds on both rations and in the two experiments increased with storage time. However, the increase in acid numbers was not always linear. Statistical analysis pointed out that the only significant ($P<.05$) increase in acid numbers occurred in drippings from 1952 birds on both rations between 12 and 16 months of storage.

Differences in peroxide values for fat in drippings from birds fed the 2 rations were non-significant. In 1952 the peroxide values for both xanthophyll and control birds increased significantly ($P<.05$) between 0 and 4 and between 12 and 16 months of storage, but those from birds reared in 1953 showed no significant increase as storage time progressed, Table 2. It is recognized that cooking per se caused the development of peroxide oxygen in the fat. However, it was observed that the initial values were much higher for the 1953 birds than for those reared in 1952. On the other hand, the increase in the mean peroxide numbers between 0 and 4 months were higher in 1952 than in 1953, Table 2. No explanation can be given for the high initial peroxide values for fat in the drippings from 1953 birds.

Objective tests. *Cooking losses.* Average total cooking losses for xanthophyll and control birds reared in 1952 and 1953 are given in Table 3. Cooking losses were not significantly affected by the ration fed. Analysis of variance indicated that in 1952 frozen storage over a 16-month period caused a significant ($P<.001$) increase in cooking losses, whereas in 1953, 16 months of storage brought about a significant ($P<.001$) decease in cooking losses. Least significant differences pointed out that in 1952 the variation in total cooking losses for the birds fed the xanthophyll could be attributed to the significantly ($P<.05$) greater losses for birds held in frozen storage for 4 months than those for fresh birds. Storage beyond 4 months did not affect significantly the total cooking losses from these birds, Table 3. The total cooking losses for 1952 birds on the control ration were not affected significantly until after 8 months of storage, Table 3. In 1953 the total cooking losses for birds on both rations were significantly ($P<.05$) less after 4 months of frozen storage than they were for fresh birds. At each storage period up through 12 months the cooking losses for the 1953 birds continued to decrease significantly ($P<.05$), Table 3. No explanation can be given for the fact that frozen storage increased the cooking losses of the birds in one experiment and decreased those of the birds in the other experiment.

Press fluid yields. Mean press fluid yields from the *pectoralis major* muscle are presented in Table 3. The incorporation of xanthophyll in the ration had no significant effect on the volume of press fluid. In general, the volume of press fluid increased significantly ($P<.001$) between 0 and 16 months of storage.

TABLE 3

Mean cooking losses, press fluid yields, and shear values

Factor	Storage time in months	Xanthophyll		Control	
		1952	1953	1952	1953
Total Cooking Losses, %	0	18.2	25.6	18.4	22.2
		*	*	ns	*
	4	22.3	29.4	19.2	26.9
		ns	*	ns	*
	8	22.2	20.9	19.9	20.9
		ns	*	*	*
	12	25.2	15.9	26.8	15.5
		ns	ns	*	ns
	16	25.1	15.7	23.1	17.5
	lsd	* = 3.34	* = 2.73	* = 3.34	* = 2.73
Press fluid ml/25 g. PM	0	8.4	8.3	8.8	8.5
		*	ns	*	ns
	4	9.5	8.6	9.7	8.8
		ns	*	ns	ns
	8	8.9	9.8	9.4	9.4
		ns	ns	*	*
	12	8.2	10.0	8.1	10.4
		ns	ns	*	ns
	16	8.9	10.7	9.4	10.9
	lsd	* = 0.76	* = 0.73	* = 0.76	* = 0.73
Shear value, lbs. PM	0	10.3	9.0	10.5	10.7
	4	12.3	12.2	9.3	14.0
	8	10.8	11.6	10.6	11.7
	12	9.8	12.7	9.7	12.1
	16	10.7	12.4	10.5	10.7

* Significant at the 5% level.
PM—*Pectoralis major* muscle.

Calculation of least significant differences for storage means indicated that in 1952 birds on both rations cooked after 4 months of frozen storage yielded significantly ($P<.05$) more press fluid than fresh birds, Table 3. Further storage of the xanthophyll fed birds did not significantly change the volume of press fluid, but the control birds yielded significantly ($P<.05$) less press fluid after 12 months than they did at 4 and 8 months of storage, and finally there was a significant ($P<.05$) increase in the volume of press fluid between 12 and 16 months of storage. In 1953 the volume of press fluids increased rather uniformly during 16 months of frozen storage.

Juiciness scores were lower for frozen than for fresh turkey. The relationship between greater press fluid and lower juiciness scores for frozen than for fresh meat was discussed by Hall *et al. (3)*. These authors pointed out that as a result of freezing there may be a decrease in the water-holding capacity of muscle tissue; thus, the press fluid yield would increase after freezing. They also postulated that the rapid removal of meat juices by chewing would soon

leave a tasteless and apparently dry residue of tissue in the mouth. Therefore, the palatability committee would score the meat low for juiciness.

Correlation coefficients for the volume of press fluid and juiciness scores were positive, but non-significant for the 1952 ($r = 0.187$) and the 1953 ($r = 0.210$) data. Goertz, Vail, Harrison, and Sanford *(2)* found the same relationship between the volume of press fluid and juiciness scores for the *pectoralis major* muscle.

Shear values. Mean shear values for the *pectoralis major* muscle are shown in Table 3. Analysis of variance indicated that there were no significant differences in tenderness, as measured by shear force, that were attributable to the feeding of xanthophyll in either 1952 or 1953. Furthermore, frozen storage had no significant effect on the shear values for this muscle. The extremely low correlation coefficient ($r = -0.033$) for tenderness scores and shear values of the *pectoralis major* muscle was non-significant for the 1952 birds. This factor ($r = -0.343$) was significant ($P<.05$), but still low, for the 1953 birds.

Histological study. There were no differences observed in the structure of the fibers in muscles from birds on the two rations. Also, the appearance of the intramuscular fat of the raw and cooked samples from the *pectoralis major* and *gluteus primus* muscles of both xanthophyll and control birds was similar. In general, most of the fat cells were small to medium in size. Many empty cells were observed in the cooked tissue of all samples, but few were found in the raw tissue. The effect of feeding xanthophyll on the amount and distribution of fat in these tissues will be discussed in another paper.

SUMMARY

Broad-Breasted Bronze turkeys were fed a basic type ration supplemented with 1% xanthophyll concentrate for 6 and 12 weeks prior to slaughter in 1952 and 1953, respectively. In both experiments these birds were compared with turkeys that received only a basic type ration. Data were collected for fresh turkey and after 4, 8, 12 and 16 months of frozen storage to measure the palatability, fat stability, and cooking losses of the turkeys, and for the histological structure of the *pectoralis major* and *gluteus primus* muscles.

In both experiments there were no significant differences in cooking losses, juiciness, and aroma of the turkeys; in the shear values for the *pectoralis major* muscle; or in the acid numbers and peroxide values for fat in the drippings from roasted half-birds that could be attributed to the inclusion of xanthophyll in the ration. Also, the differences in the flavor and tenderness scores for the birds on the two rations were small, and although at a few periods during each experiment they were statistically significant, they were not considered to be of practical importance.

In general, the *pectoralis major* muscle was scored higher in flavor than the *gluteus primus*, and the difference in the flavor of the two muscles increased with increased storage. The differences between these two muscles in juiciness and tenderness were not consistent.

Frozen storage adversely affected the aroma, flavor, tenderness, and juiciness scores for the turkeys, but the decline in juiciness and tenderness scores with storage was not linear. Shear values were not affected by frozen storage,

whereas, in general, press fluid yields from frozen turkey were greater than those from the fresh birds.

Acknowledgment

The cooperation of Dr. H. C. Fryer, Professor of Mathematics in charge of the Statistical Laboratory is gratefully acknowledged.

LITERATURE CITED

1. Criddle, J. E., and Morgan, A. F. The effect of tocopherol feeding on the tocopherol and peroxide content of turkey tissues. *Federation Proceedings*, **6**, 247 (1947).
2. Goertz, G. E., Vail, G. E., Harrison, D. L., and Sanford, P. E. Organoleptic and histological characteristics of fresh and frozen stored turkey fed high-density and low-density rations. *Poultry Sci.*, **34**, 640 (1955).
3. Hall, J. L., Kalen, J., Westerman, B. D., Mackintosh, D. L., and Vail, G. E. Keep the temperature low when storing pork. *Refrig. Eng.*, **24**, 696 (1949).
4. Hite, J. P., Kloxin, S. E., Kummerow, F. A., Vail, G. E., and Avery, T. B. Fat rancidity in eviscerated poultry. III. The effect of variations in dietary fat, ethanolamine, and chlorine on the storage quality of mature turkeys. *Poultry Sci.*, **28**, 249 (1949).
5. Hite, J. P., Kloxin, S. E., and Kummerow, F. A. Fat rancidity in eviscerated poultry. IV. The effect of variations in dietary fat, ethanolamine and choline on the characteristics of the fat extracted from turkeys. *Poultry Sci.*, **28**, 244 (1949).
6. Klose, A. A., Kummerow, F. A., Goertz, G. E., and Hanson, H. L. Effect of ethanolamine and certain dietary metabolites on the storage stability of frozen turkeys. *Poultry Sci.*, **31**, 730 (1952).
7. Kummerow, F. A., Vail, G. E., Conrad, R. M., and Avery, T. B. Fat rancidity in eviscerated poultry. I. The effect of variation in diet on the cold storage life of immature turkeys. *Poultry Sci.*, **27**, 635 (1948).
8. Kummerow, F. A., Hite, J. P., and Kloxin, S. E. Fat rancidity in eviscerated poultry. II. The effect of variation in diet on the characteristics of the fat extracted from immature turkeys. *Poultry Sci.*, **27**, 689 (1948).
9. Mecchi, E. P., Pool, M. F., and Klose, A. A. The role of tocopherol content in the stability of chicken and turkey fat. (Abs.) *Poultry Sci.*, **32**, 915 (1953).
10. Schreiber, M. L., Vail, G. E., Conrad, R. M., and Payne, L. F. The effect of tissue fat stability on deterioration of frozen poultry. *Poultry Sci.*, **26**, 14 (1947).
11. Watt, D. B., Hall, J. L., Mackintosh, D. L., and Vail, G. E. Heat stability of fat peroxides. *Food Technol.*, **3**, 206 (1949).
12. Watts, B. M., and Peng, D. Rancidity development in raw versus precooked frozen pork sausage. *J. Home Econ.*, **39**, 88 (1947).

THE EFFECTS OF OVEN TEMPERATURE ON CERTAIN CHARACTERISTICS OF BAKED SALMON[a]

HELEN CHARLEY AND GRAYCE E. GOERTZ[b]

Foods and Nutrition Department, Oregon State College, Corvallis, Oregon

(Manuscript received April 27, 1957)

Effects of oven temperature on cooking losses and palatability of baked Chinook salmon steaks have been reported *(1)*. The present paper reports an extension of the study of the effects of oven temperature to larger pieces of fish. As in the previous work, the cuts of salmon were baked at four different oven temperatures to a fixed internal temperature and evaporation, drip, and total cooking losses were determined, press fluid determinations were made on cooked salmon and the baked fish were rated for palatability by a panel of six judges. In addition, raw and cooked samples from each cut of salmon were studied histologically.

EXPERIMENTAL PROCEDURE

Preparation of the cuts of fish. Fresh Silverside salmon (Oncorhynchus kisutch) were purchased locally. The fish varied in weight from 8 to 11 pounds. Each fish was divided into four parts of approximately equal weights. The first cut extended from the region of the pectoral fin and the gills half way to the dorsal fin, the second cut ended at the ventral fin and near the center of the dorsal fin, the third cut included the area from the center of the dorsal fin and the ventral fin to near the anus, and the fourth section included the remainder of the fish to the caudal fin. In the material that follows, these parts will be referred to as A, B, C, and D, respectively.

The amount of body cavity in the 4 sections affected the size and shape of each piece, and the configuration, in turn, influenced the rate of heat penetration. The individual cuts for the eight replications ranged in weight from 651.5 grams to 1060.6 grams. The average weight of the cuts used for each of the four oven temperatures are given (Table 1).

Baking. Cuts of fish were placed on pot-roasting racks resting on shallow baking pans and were baked simultaneously in four household electric ovens at 350° F., 400° F., 450° F., and 500° F. Baked fish were removed from the oven when the internal temperature reached 70° C. (158° F.) and the rise in internal temperature after removal from the oven was noted.

Testing. As in the previous work with salmon steaks (1), cooking losses were

[a] Supported in part by a grant-in-aid from General Research, Oregon State College, Corvallis, Oregon.

[b] Present address: Department of Foods and Nutrition, School of Home Economics, Kansas State College, Manhattan, Kansas.

TABLE 1

Effects of Oven Temperature on Cooking Time for Baked Salmon[1]
Internal Temperature 70°C. (158°F.)

Oven Temperature	Maximum Internal Temperature	Weights of Roasts	Baking Time	
			Total	Per Pound
°F.	°C.	Grams	Minutes	Minutes
350	71.0	830.5	66	36
400	71.6	805.2	56	32
450	72.4	842.6	51	28
500	72.7	803.4	42	24

[1] Based on eight replications.

measured, press fluid determinations were made and the samples were evaluated organoleptically by a panel of six judges.

Two 4x4 latin square designs were used for the experiment giving 8 replications for each of the 4 oven temperature treatments. For the statistical analysis, the ranks of the fish baked at the 4 oven temperatures were transformed into normal scores, using Table XX, Fisher and Yates *(2)*.

Histological Study. Samples for histological sections were taken from both raw and cooked fish. Samples were removed from the area adjacent to those used for palatability and fixed in physiological salt and formalin solution. Longitudinal sections were cut 20-35 microns thick on a freezing microtome and stained with Van Giesen's, Harris' hematoxylin and Herxheimer's scarlet red to differentiate the collagenous connective tissue, muscle fibers and fat cells. The stained tissue was mounted in glycerine jelly.

RESULTS AND DISCUSSION

Baking time. Temperature of the fish at the beginning of the cooking period varied from 9° C. (48° F.) to 14° C. (59° F.). The time required to bake the salmon cuts varied as follows: 40 to 76 minutes at 350° F., 50 to 65 minutes at 400° F., 45 to 59 minutes at 450° F., and 39 to 46 minutes at 500° F. The average of the total baking time for the cuts baked at each of the 4 oven temperatures and the average baking time in minutes per pound are given (Table 1). The average baking time per pound per part was as follows: 27, 29, 31, and 29 minutes for parts A, B, C, and D, respectively.

Although all cuts were removed from the oven when the fish had reached an internal temperature of 70° C., there was a variable rise in internal temperature depending upon the cut of fish and the baking temperature. Cut C, which was the most compact, had the highest rise in internal temperature. The rise in internal temperature increased with increasing oven temperature (Table 1). The average rise in internal temperature for all cuts and all treatments was 1.9° C. or 3.4° F.

Appearance and behavior during baking. The 4 oven temperatures caused marked differences in the appearance of the fish as well as in its behavior during baking. At the two lower oven temperatures the cut surface of the baked fish was covered with a moist white coagulum and the fish appeared plump with little or no evidence of shrinkage. Browning of the cut surface, shrinkage of the flesh and blistering of the skin, all observed at 450° F., were quite marked at 500° F. At 500° F. the oven was spattered, the drippings were charred and the smoke was appreciable. These were less pronounced at 450° F. and were not observed at the two lower baking temperatures.

Cooking losses. Percentages of evaporation, drip, and total cooking losses from salmon baked to an internal temperature of 70° C. (158° F.) at each of the 4 oven temperatures are given in Table 2; for each of the 4 cuts in Table 3.

(1) Evaporation. Average cooking losses due to evaporation increased significantly with increasing oven temperature ($p<0.01$). Losses at the four oven temperatures and the minimum significant difference are included in Table 2. An increase in oven temperature of 100° (from 350° F. to 450° F. and from 400° F. to 500° F.) caused significant increases in the percentage of evaporation during baking. In the previous work with one inch salmon steaks differences in evaporation losses associated with oven temperature were not significant.

TABLE 2
Weight Losses During Baking at Different Oven Temperatures of Two Pound Cuts of Salmon

Internal Temperature 70°C. (158°F.)

Oven Temperature	Cooking Losses[1]		
	Evaporation	Drip	Total
°F.	%	%	%
350	13.36	0.99	14.34
400	15.54	0.56	16.14
450	16.99	0.49	17.51
500	18.25	0.38	18.64
F value	14.09**	15.77**	9.73**
Minimum significant difference at the 1% level	2.33	0.28	2.48

** $p<0.01$.
[1] Based on eight replications.

TABLE 3
Weight Losses During Baking of Different Cuts of Salmon
Internal Temperature 70°C. (158°F.)

Part	Cooking Losses[1]		
	Evaporation	Drip	Total
	%	%	%
A	16.95	0.61	17.58
B	16.65	0.60	17.26
C	16.76	0.52	17.31
D	13.78	0.68	14.48
F value	7.31**	0.83	6.04**
Minimum significant difference at the 1% level	2.33		2.48

** $p<0.01$.
[1] Based on eight replications.

Percentage of evaporation varied significantly with the part of the fish ($p<0.01$). Evaporation loss from part D was significantly less than that from the other three parts, no doubt because the former had so much skin and relatively little cut surface. In addition, evaporation loss varied significantly from fish to fish ($p<0.05$).

(2) Drip losses. The percentage of drippings decreased with an increase in oven temperature ($p<0.01$). Dripping losses at the 4 oven temperatures and the minimum significant difference are included (Table 2). The percentage of drip at 350° F. was significantly greater than that at the other 3 temperatures. Greater evaporation of moisture from the pan and greater decomposition of organic constituents could account for the smaller amount of drip from fish baked at the higher oven temperature. In the previous work with salmon steaks, the highest oven temperature resulted in a significantly higher per-

centage of drippings. Apparently, due to the shorter cooking time, the amount of drip squeezed out of the steak was more important than the weight lost by evaporation from the cooking pan in determining the weight of drip.

(3) Total. Total cooking losses increased significantly with oven temperature ($p<0.01$). The total cooking losses for each treatment and the minimum significant difference are included (Table 2). An increase from 350° F. to 450° F. and from 400° F. to 500° F. caused significant increases in total cooking losses. In the previous work with steaks, differences in total cooking losses associated with oven temperature were not significant.

Total cooking losses also varied with the part of the fish ($p<0.01$). Total losses for each part and the minimum significant difference are included (Table 3). The total cooking loss for part D was significantly less than that for the other three parts. In addition, total cooking losses varied significantly from fish to fish ($p<0.05$), as was the case in the earlier work with baked steaks.

Press fluid. The percentage of press fluid from the baked salmon did not vary significantly with oven temperature, which was also true for the steaks in the previous study.

Organoleptic tests. As was true in the earlier work with salmon steaks, analysis of the transformed scores failed to reveal any significant differences in palatability which were associated with baking temperatures. The judges detected differences in palatability among the parts of the fish, however. The average normal scores for the 4 different cuts of salmon, disregarding oven temperature, are given (Table 4). The flakiness of the fish varied significantly with the part ($p<0.01$). Minimum significant differences are included in Table 4. The judges ranked part A significantly flakier than parts C or D. They were unable to detect differences in moistness among the 4 parts. The tenderness varied significantly with the part of the fish ($p<0.05$), the judges ranking part D significantly more tender than part A. The flavor of the fish varied significantly with the part ($p<0.01$). Part C was ranked significantly

TABLE 4

Average Normal Scores for Four Salmon Cuts[1]
Internal Temperature 70°C. (158°F.)

Parts	Palatability Characteristics				
	Flakiness	Moistness	Tenderness	Flavor	Over-all Desirability
A	0.4341	0.1953	−0.3258	−0.3737	−0.3661
B	0.1282	0.2561	−0.0442	−0.1380	0.0184
C	−0.1331	−0.2083	0.0916	0.4085	0.2950
D	−0.4292	−0.2431	0.2784	0.1032	0.0527
F-value	8.80**	2.15	3.86*	9.26**	3.52*
Minimum significant difference at the 1% level	0.5174		0.5392	0.4585	0.6072

* $p<0.05$.
** $p<0.01$.
[1] Based on eight replications.

higher for flavor than parts A and B, part D significantly higher than part A. The over-all desirability of the fish varied significantly with the part ($p<0.05$). The judges ranked part C significantly higher than part A for over-all desirability.

Histological findings. The histological appearance of the fat, collagenous connective tissue and muscle fibers did not appear to be affected by the four oven temperatures. Differences were observed, however, between the raw and cooked tissues (Figures 1A and 1B).

Figure 1A. Photomicrograph of salmon tissue. (Magnification x 112.) Raw tissue with a few fat cells embedded in the band of connective tissue.

As a whole, disintegration, which was much in evidence in the raw fish, was even more pronounced in the muscle fibers of the corresponding cooked tissue. Granular material replaced sections of the striae in the disintegrated areas. Few rigor nodes were observed in any of the samples studied. The individual muscle fibers of the uncooked fish appeared separate and distinct with little evidence of connective tissue holding them together. In the cooked

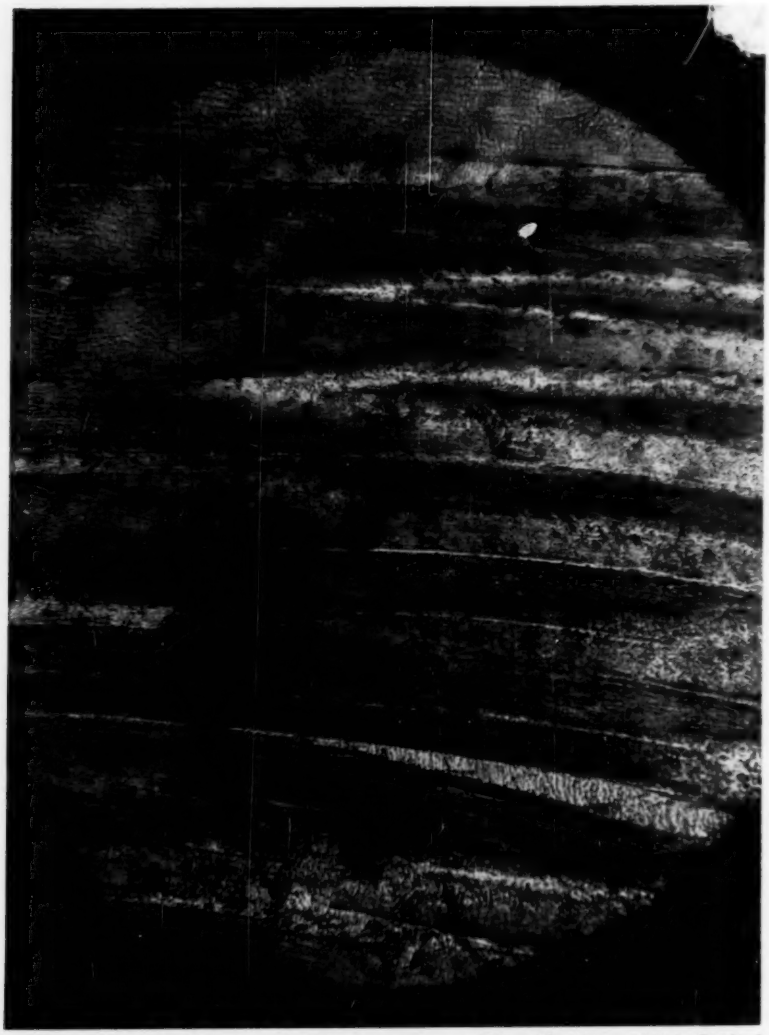


Figure 1B. Photomicrograph of salmon tissue. (Magnification x 112.) Cooked tissue showing distintegration in muscle fibres and granulated areas of connective tissue.

samples the width of the fibers appeared to increase and fill up the interfibrillar spaces. Kinks and twists apparently disappeared with baking, as fewer were observed in the cooked than in the raw tissue. The wavy fibers of the collagenous connective tissue of the uncooked fish changed to granulated and gel-like areas in the cooked samples. Less fat appeared in the cooked samples and it was more dispersed than in the raw.

Histological differences were observed among the individual fish. The amount of fat and extent of disintegration of muscle fibers varied from fish to fish and this, no doubt, affected the percent of evaporation and total cooking losses.

More pronounced histological differences were observed among the four parts of the fish, and this may account, in part, for some of the differences in palatability which the judges detected. For example, kinks and twists appeared more frequently in the individual muscle fibers of the section nearest the head and least frequently in the tail cut. These twists occurred most often near areas of masses of connective tissue. A considerable amount of kinkiness was observed in the raw collagenous tissue in part A. This was true to a lesser degree in the other sections, with the least amount occurring in part D. The smallest extent of breakdown of muscle fibers and of collagenous tissue appeared in part A and the greatest amount of disintegration in part D. It was of interest that judges found part D, which contained fewer kinks and twists and greater breakdown of fibers, significantly more tender than part A.

The amount of fat decreased slightly but progressively from part A to part D, in which the least amount was observed. Much of the fat in all of the cuts appeared to be localized in the perimysium and this was especially true in part D. However, where larger quantities of fat were present, more of it appeared between the individual muscle fibers. These fat cells were quite small compared with the medium to larger angular and irregularly shaped cells in the perimysium areas.

SUMMARY

Cut of salmon weighing approximately 2 pounds were baked to an internal temperature of 70° C. (158° F.) at oven temperatures of 350° F., 400° F., 450 F°., and 500 F°. Average baking time decreased from 36 minutes per pound at 350° F. to 24 minutes at 500° F. The third cut from each fish was the most compact and this part had a somewhat longer baking time, while the first cut nearest the head of the fish and with the large body cavity had the shortest baking time per pound.

Shrinkage of the meat and blistering of the skin was markedly less at the lower oven temperatures. The oven required extensive cleaning when the fish was baked at the higher oven temperatures while little or none was needed at the lower ones. These disadvantages to using the two higher oven temperatures were more marked for the two pound cuts of salmon than they were for the one inch salmon steaks previously studied.

A 100° F. increase in baking temperature resulted in a significant increase in evaporation and in total cooking losses, in contrast to the one inch salmon steaks previously studied where baking temperature made no difference in either. Evaporation and total cooking losses were less for part D than for

the other three parts. In addition, evaporation, drip, and total cooking losses differed significantly from fish to fish. While the amount of fat and the disintegration of muscle fibers as studied histologically also differed among fish as well as parts of fish, the baking temperature had no effect on the histological appearance of the cooked salmon. Oven temperature had no effect on the amount of press fluid or on the palatability ratings of the baked salmon. Differences in certain palatability characteristics of the baked fish were associated with the part of the fish. The cut nearest the head end ranked highest in flakiness and lowest in tenderness, while that from the tail was least flaky but most tender. Histologically, the tail cut contained the least number of kinks and twists and the greatest degree of disintegration of muscle tissue while the opposite was true for the cut nearest the head. The cut from the third quarter was ranked highest for flavor and over-all desirability, that from the head end lowest.

Acknowledgments

The authors wish to express appreciation to the following who served as judges: Alta Garrison, Andrea Mackey, Margaret Ware, and Kyriake Valassi.

Statistical work was done under the direction of Dr. Lyle Calvin, Agricultural Experiment Station Statistician, Oregon State College, Corvallis.

LITERATURE CITED

1. Charley, Helen. Effects of internal temperature and of oven temperature on the cooking losses and palatability of baked salmon steaks. *Food Research*, **17**, 136-143 (1952).
2. Fisher, R. A., and Yates, F. *Statistical Tables for Biological, Agricultural, and Medical Research*. 1938. Oliver and Boyd, London, England.

ENZYME ACTION AND OFF-FLAVOR IN FROZEN PEAS[a]

A. C. WAGENKNECHT AND FRANK A. LEE

New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.

(Manuscript received May 1, 1957)

Enzymes are believed to be responsible for the development of off-flavor in raw and underblanched vegetables during frozen storage *(1)*. A variety of enzymes have been suggested as being causative agents in flavor deterioration *(1)*, but interest has centered mainly around catalase and peroxidase. Tests for the presence of these two enzymes have long been used as criteria of adequacy of blanching of vegetables prior to freezing. Furthermore, a reasonably good correlation has been observed between the presence of residual amounts of these enzymes and extent of off-flavor in frozen vegetables. To date, however, the identity of the responsible enzymes has remained obscure.

Recently it has been shown that lipoxidase and lipase are responsible for the progressive deterioration of lipids in raw peas during frozen storage *(5, 12)*. These changes in the lipids, characterized by increases in titratable acidity, development of lipid peroxides, and loss of chlorophyll, closely paralleled the progressive development of off-flavor *(7)*. It was deemed most likely that lipoxidase and possibly lipase are primary causative agents in the production of off-flavors during frozen storage of raw peas.

Proof of involvement in off-flavor development of any or all of the above enzymes may be ascertained by a study of their action in model systems. Preparations of catalase, peroxidase, lipoxidase, and lipase of known specific activity were added to enzymatically inert blanched peas, and the samples analyzed after a suitable period of frozen storage.

This paper reports the production of off-flavors during the frozen storage of macerates of blanched peas, brought about by the addition of enzymes, and a correlated chemical study of lipid deterioration.

MATERIALS AND METHODS

Perfected Freezer peas, grown on Experiment Station plots, were harvested and processed as described earlier *(5)* and stored in 30-pound tins at –17.8° C. The blanched peas to which enzymes were to be added were thawed and blended for 2 minutes in a five-quart Waring blender with one-half their weight of water. The resultant pea slurry was divided into 100 g. lots, and the various enzyme preparations were added and well mixed by stirring. The amounts of enzymes added to blanched peas were calculated to be twice the amounts present in fresh raw peas. Four series of samples were prepared in which the 4 enzymes were added singly and in all possible combinations. Samples of blanched pea slurry with no added enzymes were included as controls in each series. Two series were used for taste test evaluation for flavor and color *(12)*, and two series were used for chemical analysis. Samples were placed in cellophane bags inside waxed cartons, frozen, and held in storage at –17.8° C.

Catalase activity was measured manometrically by the Thompson method *(11)*. Crystalline catalase (General Biochemicals, Inc., Chagrin Falls, Ohio), specific activity —0.018 mg. produced 2.5 ml. O_2, was added at the level of 17 mg. per 100 g. lot of peas. One gram of the resultant mixture produced 2.8 ml. of O_2.

---

[a] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1075.

Peroxidase was determined colorimetrically by the method of Keilin and Hartree *(2)*. Horseradish peroxidase, purpogallin number (P.N.) 533, was prepared by the method of Kenten and Mann *(3)* from horseradish roots grown on Experiment Station plots. When added at the level of 1.17 mg. per 100 g. lot of peas, this peroxidase produced a P.N. of 0.07 in the resultant mixture.

Lipoxidase activity was determined manometrically in the Warburg respirometer using linoleic acid as substrate *(12)*. Soybean lipoxidase (Nutritional Biochemicals Corp., Cleveland, Ohio) with a Q_{o_2} of 702 *(12)*, when added at the level of 42 mg. per 100 g. lot of peas, produced a Q_{o_2} of 1.65 in the resultant mixture.

Lipase activity was measured manometrically in the Warburg respirometer using tributyrin as substrate *(8)*. Pancreatic lipase (Nutritional Biochemicals Corp., Cleveland, Ohio) with a Q_{CO_2} of 607, when added at the level of 66 mg. per 100 g. lot of peas, produced a Q_{CO_2} of 7.0 in the resultant mixture.

One series of samples for chemical analysis was lyophilized in a Stokes cabinet type freezer-drying unit with the heated plates at 20° C. The dried samples were extracted for 24 hours with anhydrous diethyl ether and the ether extracts assayed for acid number and peroxide number *(4, 5)*.

The fourth series of samples was used for the determination of residual enzyme activity following frozen storage and for chlorophyll analysis *(13)*.

RESULTS

The results of this study are summarized in Table 1.

The samples of blanched peas to which enzymes had been added were subjected to taste panel evaluation for flavor and color after 3 months and again following 17 months of storage at −17.8° C. In each case evaluation of the samples was based on comparisons with the flavor and color of blanched peas with no added enzymes, which had been stored under similar conditions. The finding of lowered flavor scores after three months storage is similar to the results obtained with intact raw peas *(7)*. It may be concluded that the addition of enzymes to blanched peas did produce off-flavors ranging in intensity from mild to extremely objectionable. Mild off-flavors were observed in samples containing added catalase and peroxidase. The more objectionable off-flavors were found in samples to which lipoxidase had been added. In general the effect of the addition of mixtures of two or more enzymes was the same as the effects produced by the enzymes when added individually. The sole exception was the apparent lessening of off-flavor intensity occasioned when catalase was present in samples also containing lipoxidase or peroxidase.

Changes in color brought about by the addition of enzymes were readily discernible by visual inspection by the taste panel, and were also evidenced by chlorophyll losses measured spectrophotometrically. The normal bright green color was modified to a dull grey-green in all of the samples containing lipase. Lipoxidase-treated samples showed a slightly lower visual color score, based on a difference in saturation of color, rather than a change of hue. Catalase and peroxidase did not cause any visually detectable changes in color. However, a measurable loss of chlorophyll was sustained by treatment with any or all of these four enzymes, ranging from 2.8% to 35.8%. The greatest losses were observed in the lipase-treated samples. Spectrophotometric observations of the acetone extracts of lipase-treated peas between 435 and 443 mμ indicated an average increase of 20% in optical density in this region over that of blanched pea control samples. This was taken as evidence for the presence of pheophytin which has a characteristic peak in this region of the spectrum.

Deterioration of lipids was evidenced by increases in titratable acidity and organic peroxides in the ether extracts of lyophilized samples. Lipase produced very large increases in acid number, larger indeed than those normally encountered in frozen raw peas after prolonged storage *(5, 7)*. Catalase-treated samples showed a slight increase in acid number. Peroxidase and lipoxidase had little or no effect on the amount of titratable acid. Positive peroxide numbers were observed in the lipids from samples treated with lipoxidase and peroxidase.

Analyses of the samples for residual enzyme activity following 17 months of frozen

TABLE 1
The effect of added enzymes on quality of blanched peas

Sample[1]	Taste panel evaluation				Chemical evaluation after 17 months storage at −17.8°C.						
	3 months[2]		17 months[2]		Chlorophyll,	Acid	Peroxide	Residual enzyme activity Percentage of amount originally present			
	Flavor	Color	Flavor	Color	% destroyed	number	number	Catalase	Peroxidase	Lipoxidase	Lipase
Blanched control	7.5	8.1	7.4	8.4	0	5.4	0	0	24[3]	0	0
A	6.4	8.0	6.2	8.4	13.7	11.6	0	75	—	—	—
B	5.9	7.5	6.1	8.3	6.2	7.3	2.7	—	97	—	—
C	3.5	7.9	5.3	8.1	12.3	6.8	2.4	—	—	60	—
D	1.5	5.2	1.8	3.7	27.7	120.0	0	—	—	—	96
AB	5.0	7.4	5.8	8.4	2.8	8.6	2.9	61	127	—	—
AC	4.8	7.4	6.2	8.5	21.4	16.5	5.1	57	—	81	—
AD	1.6	4.9	2.2	3.5	25.4	120.9	0	75	—	—	89
BC	5.0	7.4	4.6	7.8	7.7	9.0	3.6	—	89	95	—
BD	1.7	4.6	2.2	3.5	33.8	119.7	Trace	—	81	—	69
CD	2.3	6.4	1.8	3.8	35.8	123.9	1.7	—	—	88	66
ABC	5.7	7.5	5.9	7.5	16.5	12.8	5.8	54	187	119	—
ABD	2.3	3.8	1.4	1.7	24.8	130.4	0	72	92	—	103
ACD	2.7	6.5	2.3	4.8	24.3	128.5	4.0	91	—	40	87
BCD	2.4	5.2	2.1	3.3	28.7	121.6	3.4	—	40	92	66
ABCD	2.6	6.5	2.9	5.1	26.7	129.2	3.7	82	76	95	98

[1] A — catalase
B — peroxidase
C — lipoxidase
D — lipase

[2] Storage time at −17.8°C.

[3] Percentage of activity of Sample B prior to storage.

storage indicated that the enzymes had in every instance retained their activity to a large degree under these conditions.

DISCUSSION

Certain limitations must be recognized in the use of model enzyme systems such as those employed in the present study. One cannot be assured of duplicating the conditions occurring in intact tissues. For example, maceration of the blanched peas in a blender resulted in appreciable aeration. The oxygen content of the macerate would be expected to be greater than that in intact peas. However, the blanched pea macerates without added enzymes were included as controls for both organoleptic and chemical evaluation, and no adverse changes in quality were detected either initially or after prolonged frozen storage.

In these studies with model systems the primary concern was with the production of quality changes rather than with absolute values of enzyme activity. The prerequisite was that the blanched pea macerates contained what was calculated to be a reasonable level of the individual enzymes prior to storage, and that sufficient enzyme had been added to insure the presence of an excess of enzyme throughout the storage period. The calculated amounts of enzymes, however, failed to produce the desired levels of enzyme activity. Consequently, larger quantities of enzymes were added prior to storage of the samples. It is quite likely that the discrepancies between the calculated amounts and those actually required were due to adsorption phenomena. Catalase, for example, is very readily adsorbed.

The off-flavors produced in this study by the addition of enzymes to blanched peas were generally similar to those occurring during the frozen storage of raw peas *(5)*. All of the samples containing added enzymes were tasted prior to freezing. No changes in flavor ascribable to the flavor of the enzyme preparations were noted, except with the lipase preparation, which imparted a distinctly different flavor to the peas. The low flavor scores of samples containing added lipase must be attributed at least in part to the flavor imparted by the lipase itself. However, these samples contained large amounts of titratable acidity, a condition which has been well correlated with objectionable off-flavor in frozen raw peas *(5, 7, 12)*.

The change in color brought about by lipoxidase action represents conversion of chlorophyll to colorless products of unknown composition *(10)*. During the manometric determination of residual lipoxidase activity the addition of linoleic acid produced additional losses of chlorophyll. At the end of the hour-long determinations the pea tissue fragments had been bleached nearly colorless.

The commercially obtained enzymes used in this study were checked for enzymatic purity, i.e., absence of the other three enzymes. A trace of catalase activity was found in the pancreatic lipase (0.5 mg. produced 0.15 ml. O_2 in the Thompson test).

The data with respect to acid numbers are rather clearcut. When lipase was present, large acid numbers resulted. Although a relatively wider range of values was obtained with peroxide numbers than with acid numbers, this was in keeping with previously published results dealing with progressive

deterioration of vegetable lipids during frozen storage *(7)*. Unpublished data from this laboratory has indicated that more than one type of organic peroxide is present in the lipids of raw vegetables following prolonged frozen storage. Hence, the absolute amounts detectable may show some variations depending upon the influence of other agents present in the individual samples. For example, the presence of peroxides in the lipids from peroxidase-treated pea samples is somewhat puzzling, for the horseradish peroxidase preparation was totally devoid of any lipoxidase activity. The possibility exists that the peroxides detected may have been due to nonenzymatic peroxidation brought about by heme-catalyzed oxidation of the lipids.

The value of the present data would have been enhanced by a study of the relative rates of formation of acids and peroxides. The current study had as its major objective the net production of quality changes which could be attributed to the addition of specified quantities of the four enzymes employed. Just how much of the changes in quality occurred during freezing is therefore, not known, but if an analogy may be drawn with studies on intact raw peas *(7)*, it would be expected that the major increases in acid number occurred during the earlier stages of storage.

The peas used in this study were given the customary 60 second blanch in boiling water, followed by cooling in tap water *(5)*. The peroxidase content of peas similarly treated was very low immediately following blanching (P.N. = 0.006), and no increase in residual peroxidase activity was apparent after eight days storage at –17.8° C. However, regeneration of peroxidase was noted in the blanched control pea samples after prolonged storage, amounting to some 24% of the original activity of the peroxidase-treated samples. The fact that this amount of peroxidase produced no off-flavors in the blanched control samples, coupled with the fact that properly blanched peas often contain residual peroxidase activity, may be taken as further evidence that peroxidase is not of prime importance in off-flavor production in frozen raw peas.

Interpretation of the data on residual enzyme activity was difficult. The purpose of having made such determinations was primarily to insure that a workable excess of the enzymes had been present throughout the storage period. Thereby, apparent non-involvement of any of the enzymes in quality changes could not be ascribed to insufficient quantity of enzyme, but rather to the non-participation of the enzyme in the sequence of reactions which produce off-flavors and other quality changes. The general decrease in residual enzyme activity found after frozen storage could have been due to interference of enzymes added to the peas, or of constituents in pea tissue, in the actual determination of enzyme activity. The two cases of increased peroxidase activity cannot be attributed solely to the regeneration of the original peroxidase present in the pea tissue because this occurred only in the presence of added catalase or catalase plus lipoxidase.

The role of lipoxidase in off-flavor development in raw peas has recently been clarified. Siddiqi and Tappel *(9)*, in a study of the oxidation products of pea lipoxidase action, reported the presence of monocarbonyls as a result of lipoxidase catalysis of the oxidation of ammonium linoleate at 0° C. They postulated that the hydroperoxides formed under these conditions were very unstable, decomposing to form aldehydic products and polymers. These oxi-

dation products were considered to be of greater importance in contributing to off-flavor than were the products resulting from autoxidation of fats during storage of frozen raw peas.

A possible explanation of how lipase may function in off-flavor production is suggested by the following observations. During the determination of residual lipoxidase activity it was observed that samples containing both lipoxidase and lipase showed considerable amounts of oxygen uptake in the absence of added linoleic acid substrate. This effect was not apparent when only lipoxidase was present, nor when catalase and/or peroxidase were present along with lipoxidase. Under the above conditions the sole source of lipoxidase substrate was the endogenous supply of lipids in the blanched peas. From the acid number determinations on these samples it was evident that considerable amounts of triglyceride had been split by the added lipase. Apparently the free fatty acids were more readily utilizable as lipoxidase substrates than the intact triglycerides. During the progressive development of off-flavor in frozen raw peas during storage, the increase in acid number invariably precedes the development of peroxides detectable in the lipids *(7)*. Thus lipase may serve as an adjunct to lipoxidase in off-flavor development through a labilization of substrates.

SUMMARY

The addition of enzymes to blanched peas resulted in the production of off-flavors following frozen storage. Lipoxidase-treated samples were moderately off-flavored. Catalase and peroxidase produced only mild changes in flavor. Lipase-treated samples possessed disagreeable off-flavors, which must be attributed in part to the flavor imparted by the lipase preparation itself.

Changes in green color were produced by the addition of all four of these enzymes. Lipase caused the greatest losses of chlorophyll, evidenced by the formation of pheophytin.

Deterioration of lipids was caused by lipase and lipoxidase, resulting in large increases in acid number and in development of peroxides.

A possible mechanism by which lipase may function in off-flavor development has been proposed.

LITERATURE CITED

1. Joslyn, M. A. Enzyme activity in frozen vegetable tissue. *Advances in Enzymology*, **9**, 613 (1949).
2. Keilen, D., and Hartree, E. F. Purification of horseradish peroxidase and comparison of its properties with those of catalase and methaemoglobin. *Biochem. J.*, **49**, 88 (1951).
3. Kenten, R. H., and Mann, P. J. G. A simple method for the preparation of horseradish peroxidase. *Biochem. J.*, **57**, 347 (1954).
4. Lee, F. A. Chemical changes taking place in the crude lipids during the storage of frozen raw vegetables. *Food Research*, **19**, 515 (1954).
5. Lee, F. A., and Wagenknecht, A. C. On the development of off-flavor during the storage of frozen raw peas. *Food Research*, **16**, 239 (1951).
6. Lee, F. A., Wagenknecht, A. C., and Graham, R. Influence of vining on the development of off-flavor in frozen raw peas. *Food Research*, **21**, 666 (1956).
7. Lee, F. A., Wagenknecht, A. C., and Hening, J. C. A chemical study of the progressive development of off-flavor in frozen raw vegetables. *Food Research*, **20**, 289 (1955).

8. MARTIN, H. F., AND PEERS, F. G. Oat lipase. *Biochem. J.*, **55**, 523 (1953).
9. SIDDIQI, A. M., AND TAPPEL, A. L. Catalysis of linoleate oxidation by pea lipoxidase. *Arch. Biochem. Biophys.*, **60**, 91 (1956).
10. STRAIN, H. H. Unsaturated fat oxidase: specificity, occurrence and induced oxidations. *J. Am. Chem. Soc.*, **63**, 3542 (1941).
11. THOMPSON, R. R. Simplified apparatus for catalase determination. *Ind. Eng. Chem., Anal. Ed.*, **14**, 585 (1942).
12. WAGENKNECHT, A. C., AND LEE, F. A. The action of lipoxidase in frozen raw peas. Food Research, **21**, 605 (1956).
13. WAGENKNECHT, A. C., LEE, F. A., AND BOYLE, F. P. The loss of chlorophyll in green peas during frozen storage and analysis. *Food Research*, **17**, 343 (1952).

EFFECT OF DEGREE OF FINISH ON DIFFERENCES IN QUALITY FACTORS OF BEEF

MARION SIMONE, FLOYD CARROLL AND M. T. CLEGG

Departments of Food Technology and Animal Husbandry, University of California, Davis, California

(Manuscript received May 17, 1957)

In beef production, the animal characteristics which contribute greatly to the grade and subsequent price of the meat include the degree of finish. This criterion is measured by the fat cover of the carcass and degree of fat marbling in the muscle.

A number of investigators have endeavored to determine the fat content in beef necessary to establish the optimum quality level with respect to tenderness, juiciness and flavor factors. In an earlier publication *(6)*, the authors have reviewed these studies, with a resulting indication of a high degree of variability among the findings.

In an attempt to establish whether different degrees of finish in animals resulted in differences in meat quality, a three year study was undertaken by the Departments of Animal Husbandy, Food Technology and Home Economics of the University of California at Davis.

Included in the following report is an evaluation of differences in eating quality factors in beef attributable to differences in fat content and subsequent U.S.D.A. grade; the application of a sensory difference method (triangle) of evaluating a food product of somewhat extreme variability; and a comparison of this sensitive technique with a quality judgment procedure (scoring).

EXPERIMENTAL PROCEDURE

Animals. For the three-year study, a total of 48 Hereford steers from the range herd of the University of California were used. Each year, 16 animals above average in uniformity of breed, age and quality were put into feed lots at the Davis campus. Group I (8 animals) was fed a low concentrate ration whereas Group II (8 animals) was fed a high concentrate ration. For each year's experiment both groups received the same concentrate consisting of 65% ground barley, 20% molasses beet pulp and 15% ground oats. Average ages, weights, % concentrate, performances, slaughter grades and estimate of carcass composition of the animals in each of the 3 trials are recorded in Table 1.

At the end of the fattening period, 4 animals were slaughtered each of 4 consecutive weeks at a local packing house. Two animals were selected from Group I on the basis of a live slaughter grade of good and two animals, from Group II, with a live slaughter grade of choice. The carcasses were graded by a U. S. Department of Agriculture inspector and aged for 11 days before butchering and testing. The composition of the carcass was estimated from density measurements of the 12th rib and also from actual physical separation of this cut into lean, fat and bone; both methods having good reliability according to Lofgreen and Garrett *(4)* and Hankins and Howe *(3)*. Muscular fat (marbling) determinations were made on the 12th rib, right side, using the ether extract procedure.

Meat cuts. Butchering of the carcasses in the first two trials was done by a commercial firm[a] following specific instructions of the experimenters. The University cafeteria meat department butchered the carcasses for the third trial, following the same instructions.

[a] DelPero-Mondon Meat Co., Marysville, California.

Two three-rib roasts (6-7-8- and 9-10-11) and two 3-inch top round roasts from both sides were used in the tests.

Testing designs and procedure. A modified triangular design was used for the sensory difference method of testing the two groups. Only the roasts from the left sides of the paired animals were used. The design included the checking of the duplicate samples; indication of the flavor or texture or both factors as the judge's criterion of differentiating; and a designation of better meat flavor and more tenderness to the odd or duplicate samples.

Approximately 24 judges participated in each trial. These people were not specifically trained in meat-difference testing, but all had considerable experience in sensory difference testing of many foods and beverages, for both flavor and texture qualities. There was an approximate turn-over of 40% of the judges from one trial to the next.

Since two pairs of animals were slaughtered each of 4 consecutive weeks, a total of 8 comparisons was made each week. The 4 top round comparisons were made on 4 consecutive mornings (10:45 A.M.) and the rib roasts on the same afternoons (3:45 P.M.). The roasting procedure was used for preparation of both cuts of meat.

The top rounds, varying from 3½ to 5 pounds, were roasted, uncovered, in aluminum pans at 300° F. to an internal temperature of 150° F. This required from 1 to 2 hours. Rib roasts ranging from 7 to 11 pounds were similarly roasted, requiring from 4 to 5 hours.

To avoid appearance biases in the judges' selection of the duplicate samples, extreme care was followed in the preparation of the meat for serving. Each set contained pieces carefully matched with regards to location in the muscle, size and shape. The *longissimus dorsi* and *spinalis dorsi* muscles of the rib roasts and the *semi-membranosus* and the *adductor* muscles of the top round roasts were used. The pieces were trimmed of fat and served in sizes of approximately 2 sq. inches by ¼ to ⅜ in. thick. The pieces were arranged in shallow enamel pans according to muscle location and kept warm on a steam bath during the tasting session, which extended from 20 to 30 minutes after carving.

Samples for each triangle set were arranged randomly opposite code numbers on a warmed, white serving plate. Each participating judge evaluated his set of samples under low, red illumination in a room equipped with individual taste booths. No specific instruction was given to the panel members regarding tasting mechanics.

In order to evaluate the triangular design as a procedure for testing differences in meat, published and unpublished data from other studies reported by the authors is included *(6, 7)*. These data were obtained from scoring tests in which muscles from the same cuts of meat from the right sides of the same paired animals used in the above tests were compared with beef from two other animals subjected to other treatments (hormone and/or type of ration).

A 14-member panel of selected judges scored 5 samples of meat for tenderness, juiciness, and flavor using a 7-point scale for each factor: 7 indicating *very desirable*; 5, *desirable*; and 2 *just acceptable*.

Methods of cooking, matching slices and serving procedures were identical with those described for the triangle test. However, meat pieces used in the scoring test were larger than those in the triangle test. This was possible because of the design requiring fewer samples and a smaller number of judges.

RESULTS AND DISCUSSION

Information in Table 1 illustrates the differences in average age, grade, and degree of fatness of the animals used in each of the three trials. The differences in quality grades of the two groups increased each year. The greatest difference occurred in trial 3 because the extent of finish was easier to control in younger animals being fed over a longer period resulting in more fat deposition in the muscle of the high-concentrate-fed animals of trial 3. Because of this effect there was a much wider difference between the fat content of the eye muscle of the two groups of trial 3 than between these

TABLE 1
Animal history and feed-lot performance

	Trial 1 Average days on feed—97		Trial 2 Average days on feed—185		Trial 3 Average days on feed—207	
	Group I	Group II	Group I	Group II	Group I	Group II
No. of steers	8	8	8	8	8	8
Initial age—mos. (avg.)	27	27	20	20	10	10
Initial weight—lbs. (avg.)	943	937	831	833	547	547
% concentrate in ration	24	54	22	59	16	63
Average daily gain—lbs.	1.8	2.4	1.8	2.4	1.6	2.1
Average daily food consumption—lbs.	26.1	28.7	27.3	25.6	17.4	18.5
Feed per 100 lbs. gain—lbs. (avg.)	1450	1196	1476	1054	1088	881
U.S.D.A. slaughter grade:						
Choice—no. of animals	1	3	3	8	0	7
Good—no. of animals	6	5	4	0	3	1
Commercial—no. of animals[1]	1	0	1	0	5	0
Average fat content of carcass—%	33.3	35.6	34.6	42.6	30.3	38.3
Average fat content of eye muscle—%	2.4	2.6	4.4	4.8	5.5	11.5
Average moisture content of eye muscle—%	75.3	74.8	75.3	74.1	74.8	73.1

[1]These animals would probably have graded "standard" under the most recent grade classification system.

groups in trials 1 and 2. These variations in differences from one trial to the next are reflected in the results of the palatability difference evaluations by both the triangle and scoring procedures (Table 2).

In each trial, the judges significantly identified the duplicate samples in the triangle tests (Table 2A). However, those judges who were able to identify the duplicate samples were more in agreement with respect to the more tender and flavorful sample with each successive trial. With regard to the criterion of differentiating, approximately ⅔ of the judges indicated both flavor and tenderness, and the others were almost equally divided between flavor or tenderness. In the analysis, only results of correct separations were included in the quality factor designations. In those cases where the judges used only one criterion of differentiation, 50% credit was assigned to each group for the more desirable quality level of the other factor.

The attribution of the more desirable quality level in each of the factors was directed toward Group II with panel agreement increasing each year. This trend is more noticeable in the top round cuts than the rib roasts. The differences in the tenderness and flavor factors, however, were not highly significant until the third trial, in either top round or rib roast comparisons.

TABLE 2
Results of Triangle and Scoring Methods

A. Triangle (Chi Square Analysis)

Year	No. of Tests	No. of Correct Separations	No. indicating more desirable level			
			Tenderness		Flavor	
			Group I	Group II	Group I	Group II
Rounds:						
1954	214	125[a]	55.5	69.5	57.5	67.5
1955	275	128[a]	53.5	74.5	49	79[1]
1956	327	156[a]	56	100[a]	54	102[a]
Ribs:						
1954	294	140[a]	61.5	78.5	57	83[1]
1955	303	148[a]	64	84	62.5	85.5
1956	317	149[a]	59	90[1]	52	97[a]

B. Scoring (Analysis of Variance)

	No. of Comparisons	Tenderness		Juiciness		Flavor	
		Group I	Group II	Group I	Group II	Group I	Group II
Rounds:							
1954	234	3.83	4.14[a]	4.30	4.50	4.27	4.59[a]
1955	243	3.99	4.43[a]	3.83	4.31[a]	3.95	4.55[a]
1956	154	4.04	4.75[a]	3.77	4.32[a]	4.26	4.58[a]
Ribs:							
1954	225	5.50	5.59	5.19	5.27	5.38	5.35
1955	226	5.50	5.37	4.76	4.89	5.03	5.09
1956	155	5.55	5.89[a]	4.93	5.21[a]	4.95	5.35[a]

[1] .05 probability level of significance
[2] .01 probability level of significance
[3] .001 probability level of significance

In this trial greatest differences in carcass grade and intramuscular fat were obtained.

These same trends are apparent from results of the scoring procedure (Table 2B). For the reader's information, the analysis of these results of the scoring procedure utilized individual judges' scores as compared to those reported previously *(6)*, which were based on panel average scores of the eight animal replications as the variants. Thus, by using the individual judges' scores, the larger number of degrees of freedom permitted a more real comparison with the large numbers used in the determination of the chi square values from the triangle test data.

The scoring procedure of evaluation of the tenderness and flavor factors established highly significant differences in the top round roasts in all three trials. Only in the third trial, however, were there significant differences in the rib roasts. The juiciness factor, evaluated only by the scoring technique, showed similar trends.

No differences in results were obtained by either method of evaluation (triangle and scoring) with regard to the two muscles used in either cut. Variations in degree of juiciness, tenderness and desirable flavor were observed. These intramuscular variations have been reported by Ramsbottom *et al. (5)*.

In Table 3, data from the three trials were integrated with respect to grade comparisons. The triangle panel showed more consistency in assigning more of the attributes to Group II when the "choice" grades were compared with either "good" or "commercial," than with "choice." When "good" grades of Group II were compared with either "good" or "commercial," of Group I, then panel's ability to differentiate quality in the triangle test was lessened. In the scoring procedure, assignment of the more desirable level of the quality factors was directed toward Group II in the top round evaluations, and generally reversed to Group I in the rib roasts when these "good" and "commercial" comparisons were made. These grading trends are in line with the differences in fat content and eye muscle fat.

Considering the U.S.D.A. grading system *per se*, both panels demonstrated increasing ability to differentiate among the quality factors of the two groups when "choice" grades were compared with consecutive lower-grade steps. In these experiments the differences between "good" and "commercial" grades did not clearly reflect differences in the eating quality factors.

Thus, it appears that steers fed to a high degree of finish as required by the U.S.D.A. Choice grade most often produce a more tender and flavorful beef. There appears to be a point in the degree of finish where more consistent differences in eating quality are observed. Below a certain fatness, fewer differences are found. Thus, there may be lesser quality advantages between the "good" and "commercial" grades in contrast to definite advantages for "choice" over "good," provided the animals are uniform except for degree of fatness.

In the first two trials, where the animals were more comparable with respect to age and per cent concentrate in the diets, the resulting differences in carcass grades were not large nor were there great differences in scoring. In the third trial wider differences in grade and finish were obtained. In this

TABLE 3

Comparison of Pairs by Grade Differences
Combined Three Years

Grade Comparisons					Triangle						Scoring					
							Tenderness		Flavor		Tenderness		Juiciness		Flavor	
Group I	Group II	No. of pairs	Fat[4] Det.	Fat[4] Musc.	No. of tests	Correct Seprs.	I	II	I	II	I	II	I	II	I	II
Rounds:																
Choice	Choice	4	4.5	−.05	124	63[3]	30	33	30.5	32.5	4.20	4.28	4.14	4.35	4.21	4.64
Good	Choice	9	6.6	3.05	329	158[3]	62	96[2]	55.5	102.5[3]	3.94	4.45[3]	3.90	4.38[3]	4.10	4.66[2]
Comm.	Choice	5	8.9	7.73	201	99[3]	32.5	66.5[3]	37	62[1]	3.73	4.84[3]	3.99	4.40[2]	4.19	4.51[3]
Good	Good	4	2.0	−.16	79	47[3]	16	31[1]	16.5	30.5	3.84	4.15[1]	4.11	4.35[1]	4.21	4.52[1]
Comm.	Good	2	8.2	1.79	83	42[2]	24.5	17.5	21	21	4.43[1]	4.02	3.66	4.44[3]	4.14	4.42
Ribs:																
Choice	Choice	4	4.5	−.05	160	76[3]	39.5	36.5	37	39	5.60	5.44	5.35	4.98	5.28	5.27
Good	Choice	9	6.6	3.05	325	156[3]	67	89	64.5	91.5[1]	5.39	5.63[1]	4.76	5.18[3]	5.08	5.28[3]
Comm.	Choice	5	8.9	7.73	196	102[3]	36	66[2]	32.5	69.5[3]	5.52	5.87[3]	4.90	4.94	4.91	5.33[2]
Good	Good	4	2.0	−.16	156	70[3]	26.5	43.5	27.5	42.5	5.55	5.45	5.11	5.03	5.35	5.19
Comm.	Good	2	8.2	1.79	77	33	15.5	17.5	10	23[1]	5.97[1]	5.62	4.94	5.42[1]	5.09	5.22

[1] .05 probability level of significance.
[2] .01 probability level of significance.
[3] .001 probability level of significance.
[4] Difference of Group II from Group I.

case the judges consistently and significantly favored meat from the higher finished group with respect to all the factors tested.

In order to determine if a relationship existed between the differences in scores and fat or moisture content of the two groups, correlation coefficients were determined (Table 4). Although all of the relationships with fat content are positive, there appears to be very meager evidence of a significant relation-

TABLE 4
Correlation Coefficients
% Fat (density and eye muscle) and % moisture difference vs. average score difference

Density	Rounds	Ribs
Tenderness	.037	.267
Juiciness	.298	.247
Flavor	.024	.374
Eye Muscle		
Tenderness	.314	.319
Juiciness	.079	.178
Flavor	.437[1]	.542[2]
Moisture		
Tenderness	−.273	.096
Juiciness	−.137	−.212
Flavor	−.219	−.027

[1] .05 probability level of significance
[2] .01 probability level of significance

ship. The percentage of fat in the eye muscle, however, correlates better than total fat with tenderness and flavor. This observation is in agreement with results of studies reported by Cover, *et al. (1)*. The correlations indicate that the flavor factor is more closely correlated with intramuscular fat or "marbling" than with total fat. Per cent moisture appears to affect the quality factors inversely, but again, no striking correlations exist. Correlations of per cent fat differences with the triangle data did not reveal significant relationships in any case.

Although evaluation of age differences was not made by direct comparison, the scoring data in Table 2B indicates that tenderness in both rounds and ribs increased with decreasing age of the animals. The juiciness and flavor factors showed no specific trends.

Although the results of this laboratory study would not necessarily reflect the more practical consumer responses to the same variables, very good agreement between the laboratory panel and a consumer panel was obtained in trial 3. Dunsing *(2)* obtained preferences for Group II over Group I when paired steaks from each of the groups were evaluated by a household consumer panel.

TRIANGLE vs. SCORING PROCEDURE

Results reported in this study indicate that the triangle design applied to

meat products compared somewhat favorably with the scoring procedure. However, without the modification of requesting further information about the samples, the use of this design could result in meaningless conclusions. With a food product of such variability, correct identifications can be made without specific reference to those factors under study. This can be seen in Table 1A where highly significant separations were obtained in each trial without significant quality assignment.

The scoring procedure, in general, was more sensitive in establishing significant quality differences, especially in the evaluation of the top round roasts. This may be partially attributable to the fact that the panel had been more carefully selected for meat difference testing and also the actual number of judgments used in the analysis of quality differences was slightly larger in the scoring procedure, due to the elimination of those incorrect-separation responses in the triangle test.

From observations of comments made by the triangle-testing judges, factors other than those investigated were sought in selecting the duplicate samples. Thus, it appears that in products of considerable variability, specific factors under study may be masked by uncontrollable factors, with resultant loss of specificity. In a scoring type test, these factors are more easily overlooked or ignored by the judges since there is not a strong psychological drive for a "right" answer.

SUMMARY

Differences in quality factors of beef varying in degree of finish and age were determined by laboratory panels using triangle and scoring procedures. Results of the three-year study indicate that differences in tenderness, juiciness and flavor became more apparent with increasingly wider differences in degree of finish and carcass grade. The relationship of % carcass fat with quality scores did not reveal striking correlations. Flavor, however, appears to be associated with intramuscular fat.

Greatest differences in eating quality were found between "choice" and "good," and "choice" and "commercial" grade comparisons. When greatest differences in intramuscular fat (marbling) occurred, the taste panel consistently and significantly assigned the more desirable level of quality to the meat from those carcasses showing the most marbling.

The applicability of triangle testing to products of a high degree of variability does not appear to be as sensitive in evaluating specific factors as the scoring procedure, although the results of the two procedures are in agreement. Other characteristics of the triangle test contribute to its questionable usage.

Acknowledgments

The authors wish to express sincere appreciation to Mr. Walter Maslan for his assistance in the butchering of the carcasses, and to the California Cattlemen's Association and California Cattle Feeder's Association for financial assistance.

LITERATURE CITED

1. Cover, Sylvia, Butler, O. D., and Cartwright, T. C. The relationship of fatness in yearling steers to juiciness and tenderness of broiled and braised steaks. *J. Animal Sci.*, **15**, 464-472 (1956).

2. DUNSING, MARILYN. Visual and eating preferences of consumer household panel for beef of different grades. (In preparation for publication.)
3. HANKINS, O. G., AND HOWE, P. E. Estimation of the composition of beef carcasses and cuts. U. S. Dept. Agr. *Tech. Bull. 926* (1946).
4. LOFGREEN, G. P., AND GARRETT, W. N. Creatinine excretion and specific gravity as related to the composition of the 9, 10, 11th rib cuts of Hereford steers. *J. Animal Sci.*, **13**, 496-500 (1954).
5. RAMSBOTTOM, J. M., STRANDINE, E. J., AND KOONZ, C. H. Comparative tenderness of representative beef muscles. *Food Research*, **10**, 497-509, (1945).
6. SIMONE, MARION, CARROLL, FLOYD, HINREINER, ELLY, AND CLEGG, M. T. Effect of corn, barley, stilbestrol and degree of finish upon quality of beef. *Food Research*, **20**, 521-529 (1955).
7. SIMONE, MARION, AND CLEGG, M. T. Effect of methods of stilbestrol administration on quality factors of beef. (In preparation for publication.)

EFFECT OF GAMMA RADIATION ON BACTERIAL SPORES THAT HAVE UNDERGONE GERMINATION INDUCED BY TREATMENT WITH L-AMINO ACIDS[a,b,c]

B. KAN, S. A. GOLDBLITH, AND B. E. PROCTOR

Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Mass.

(Manuscript received May 14, 1957)

The first study of the ultra-rapid germination of spores induced by simple chemicals was performed by Hills *(6)*, who isolated adenosine as the active principle in yeast extract that induced germination in spores of *Bacillus anthracis*. Using as an index of germination the loss of heat resistance as determined by viable counts, he found that 2 micromoles (μM) of adenosine in his basal medium (a gelatin hydrolysate with added tyrosine) gave 90% germination after 30 minutes at 35° C. The adenosine alone was ineffective. He also noted that an increase in the concentration of the bacterial suspension caused a decrease in the rate of germination.

In a later paper *(7)*, Hills reported that 0.5 millimole (mM) of L-alanine and 0.5 mM of L-tyrosine with 0.002 mM of adenosine greatly stimulated germination without the presence of other amino acids. He found further that L-alanine was not replaceable by related compounds and that its action was greatly inhibited by small amounts of D-alanine. This inhibition could be reversed by increasing the proportion of the L-isomer. Tyrosine, however, was replaceable by its D form as well as by other structural analogues.

Hills tested 17 other amino acids without finding significant stimulation of germination and determined that of the three compounds, alanine, tyrosine, and adenosine, L-alanine was present in all cases where highly significant stimulation of germination was observed. This research was made on strains of *Bacillus subtilis*, *Bacillus cereus*, and *Bacillus megatherium* as well as on a total of 4 strains of *Bacillus anthracis*.

Stewart and Halvorsen *(11)*, in their initial paper on alanine racemase, describe a procedure that resulted within 5 minutes in the complete germination of a suspension containing 10^8 spores per ml. of *Bacillus cereus* var. *terminalis*. To induce this germination they used a medium consisting of 6 mM of L-alanine and 0.12 mM of adenosine at 30° C. and pH 7. Hitzman, Zoha, and Halvorsen *(9)* and Halvorsen *(4)* describe studies on the germination of spores of *Clostridium roseum* and *Clostridium botulinum* as induced by L-alanine, L-phenylalanine, and L-arginine with 0.05% sodium thioglycollate. They estimated that there was from 95 to 100% germination of 5×10^8 spores in from 2 to 15 minutes.

One major problem that impedes progress in the use of irradiation as a commercial process in the food industry is the development of radiation off-flavors. The incidence of these off-flavors is related to the magnitude of the

---

[a] Contribution No. 309 from the Department of Food Technology, Massachusetts Institute of Technology, Cambridge.

[b] Presented at the Seventeenth Annual Meeting of the Institute of Food Technologists, Pittsburgh, Pa., May 14, 1957.

[c] Part of a thesis submitted by B. Kan in partial fulfilment of the requirements for the degree of Ph.D. in the Department of Food Technology, Massachusetts Institute of Technology.

delivered irradiation dose. The radioresistance of vegetative cells is considerably lower than that of the corresponding spore forms, but if it were possible to insure the rapid germination of over 99% of a given spore population, the total irradiation dose required for sterilization would be reduced and the concomitant production of off-flavors would similarly be reduced. This investigation seeks to apply ultra-rapid germination induction procedures to the sensitization of bacterial spores to irradiation.

MATERIALS AND METHODS

Spores used and their treatment. The spoilage organisms studied were one anaerobic and one aerobic species of bacteria, namely, Cameron's putrefactive anaerobe No. 3679 and *Bacillus cereus* var. *terminalis*. Prior to any experimental observations, stock suspensions of spores of these organisms were pasteurized by heating for 7 minutes in boiling water. Following pasteurization, some samples of the heat-shocked spores were treated by being held in germination media of various compositions that included some amount of an l-amino acid. The exact composition of each germination medium will be specified, as required, in the discussion of results. In all cases the amino acid solution was added to an equal volume of spore suspension containing glucose, sodium thioglycollate, or adenosine (if any) added as one drop of a concentrated solution. After a suitable holding period, generally at room temperature, the spore suspension in the germination medium was either repasteurized, irradiated, or plated to secure a control count. In any given experiment the spores used in each group were obtained from a diluted suspension of 1 ml. of a stock suspension stored in a refrigerator.

Irradiation. In these experiments all irradiations were performed as follows. A 3-ml. sample of the test bacterial spore suspension in its germination medium was sealed in a 5-ml. glass ampoule. The ampoule was then irradiated with a dose of 600,000 rep of gamma rays from a cylindrical Cobalt-60 source. The design of this source has been described by Hayner and Proctor *(5)*.

Criterion of germination. In these experiments the loss of heat resistance was considered to be the criterion of germination. The pasteurization or heat-shock procedure of holding the spores in boiling water for 7 minutes was sufficient to inactivate any spores that might have germinated. The viable organisms that survived pasteurization after having been held in the germination medium were considered to have remained in the spore state. Observations by Powell *(10)*, by Church *et al. (2)*, and by Hachisuka *et al. (3)* have shown that stainability, thermolability, turbidity, and appearance of translucent areas as viewed by the electron microscope serve as criteria of spore germination. In these experiments the development of thermolability was selected as the sole criterion of germination.

Counting procedure. Viable counts were made with the use of Andersen's medium *(1)* for the P.A. No. 3679 spores and Difco Nutrient Agar for the *B. cereus* spores. Incubation was at 37° C. for 48 hours for P.A. No. 3679 and 30° C. for 24 hours for *B. cereus*. The anaerobic procedure used differed from that of Andersen as follows: Test tubes were prepared from 200 mm. lengths of 12 mm. Pyrex tubing and were used for culturing. The 5% bicarbonate solution was added directly to the agar just before pouring, and the agar was poured into the culture tubes after their inoculation with 1 ml. of diluted spore suspension. Molten vaseline stratification was used to promote anaerobiosis.

Treatment of the data. In most of these experiments, differences of less than one log cycle between mean values of groups were not considered significant. In all cases arithmetic mean values were determined for each group of samples and their duplicate plate counts or triplicate tube counts. In one experiment where it became necessary to compare two values that differed by a factor of only 2, the standard deviations of the groups were compared and the standard deviations of the ratios of these distributions divided by the control mean values were calculated. It was decided that the difference between these ratios would be significant if the ratios were more than three standard deviations apart.

RESULTS AND DISCUSSION

Observations on P. A. No. 3679. Because the ultra-rapid germination requirements of different species (and perhaps strains) of bacterial spores may vary, it was first necessary to establish conditions that would cause spores of this culture of P. A. No. 3679 to germinate. It was found that a concentration of 50 mM of L-arginine, 0.05% sodium thioglycollate, and 10 mM of glucose would, in less than 2 minutes, induce germination of over 99% of a suspension of the culture of the P. A. No. 3679 spores used in this research. When this treatment to induce rapid germination of P. A. No. 3679 spores was used, it was found that the survival of the treated spores following irradiation with 600,000 rep of gamma rays was twice as great as that of untreated spores irradiated in water suspension (Experiment A, Table 1). This was contrary to expectations, since by the criterion of heat lability it was shown that 95% of the spores had germinated after treatment, and one would normally expect

TABLE 1

Effect of gamma rays[1] on viability of treated and untreated spores of P.A. No. 3679

Treatment of spores[2]	Dilution factor	No. of tubes	Mean viable count	Standard deviation	Mean survivors (%)
Experiment A					
Untreated	10^9	24	105	14.57	....
Untreated; irradiated	10^7	24	28	13.52	2.6
Treated	10^9	12	91	11.93	....
Treated; repasteurized	10^7	12	44	11.38	4.8
Treated; irradiated	10^9	24	47	26.5	5.2
Experiment B					
Untreated	10^9	24	102	11.05	....
Untreated; repasteurized	10^9	27	65	6.96	63.7
Untreated; irradiated	10^9	24	50	11.62	4.9
Treated	10^9	24	99	8.83	....
Treated; irradiated	10^9	12	96	12.65	9.4
Treated; repasteurized	10^9	12	1	[3]	0.1

[1] Irradiation dose: 600,000 rep.
[2] Treated with 100 mM/1 of L-arginine, 0.05% sodium thioglycollate, and 10 mM/1 of glucose.
[3] No standard deviation here; all tubes gave counts of 0 to 1.

TABLE 2

Protective action of sodium thioglycollate against the effects of gamma radiation[1] on spores of P.A. No. 3679

Treatment of spores[2]	Dilution factor	No. of tubes	Mean viable count	Standard deviation	Mean survivors (%)
Experiment C					
Untreated	10^{8}	24	96	12.12	—
Untreated, irradiated, then plus compounds[2]	10^{8}	24	80	20.1	8.2
Untreated; irradiated	10^{7}	12	92	13.16	9.4
Glucose alone; irradiated	10^{7}	12	68	9.72	6.9
Thioglycollate alone; irradiated	10^{7}	12	36	6.04	36.8
Arginine alone; irradiated	10^{7}	12	57	13.34	5.8

[1] Irradiation dose: 600,000 rep.
[2] Treated with mixture of glucose (10 mM/1), thioglycollate (0.5 g/1), and arginine (100 mM/1).

vegetative cells to have an increased radiosensitivity. The experiment was repeated (Experiment B, Table 1) with the same results.

In the belief that some element of the germination medium present during irradiation exercised a protective effect on the irradiated spores, it was decided to irradiate spores in the presence of the separate components of that medium. The results, summarized in Table 2, demonstrate that sodium thioglycollate alone was responsible for the observed protective effect.

Efforts to reduce the concentration of thioglycollate 10-fold and 100-fold in the germination medium resulted in poor germination of the spores. Attempts to germinate 100-fold higher concentration of spores followed by 100-fold dilution prior to irradiation were also not successful because the spores in the thicker suspension germinated less readily than those in the more dilute suspension.

To demonstrate that a radiation sensitization effect had occurred, a comparison was made of the viable counts for treated spores and for untreated (control) spores of P. A. No. 3679. The control spores were in a suspension containing an amount of sodium thioglycollate equal to that used with the treated spores (0.5 g. per liter). The data are summarized in Table 3. The standard deviations of the survival fraction $S_{\frac{x}{y}}$ were calculated by the formula:

$$S_{\frac{x}{y}} = \frac{\bar{y}^2 S_x^2 + \bar{x}^2 S_y^2}{\bar{y}_4}$$

where $\bar{y}$ is the mean count for the unirradiated samples, Sy is the standard

deviation of the distribution of the unirradiated samples, $\bar{x}$ is the mean count for the irradiated samples, and Sx is its standard deviation. $S_{\frac{x}{y}}$ is then the standard deviation of the survival fraction.

By applying these calculations to the data shown in Table 3, the following values are obtained:

Expt.	Treatment	Ratio of x:y	Standard deviation
D	Untreated	0.165	0.0186
D	Treated	0.080	0.0100
E	Untreated	0.160	0.0009
E	Treated	0.080	0.0076

TABLE 3

Demonstration of a radiation[1] sensitization effect on spores of P.A. No. 3679

Treatment of spores[2]	Dilution factor	No. of tubes	Mean viable count	Standard deviation
Experiment D				
Untreated	10^4	12	49.7	3.13×10^4
Untreated; repasteurized	10^4	12	34.8	
Untreated; irradiated	10^3	12	80.8	6.22×10^3
Treated	10^4	12	49.2	5.01×10^4
Treated; repasteurized	10^3	12	30.4	
Treated; irradiated	10^3	12	39.7	3.09×10^3
Experiment E				
Untreated	10^4	12	50.5	2.43×10^4
Untreated; repasteurized	10^4	12	37.4	
Untreated; irradiated	10^3	12	80.6	2.64×10^3
Treated	10^4	12	50.6	2.76×10^4
Treated; repasteurized	10^3	12	30.6	
Treated; irradiated	10^3	12	40.2	3.15×10^3

[1] Irradiation dose: 600,000 rep.

[2] Untreated samples contained 0.05 g/l of sodium thioglycollate; treated samples contained 100 mM/l of L-arginine, 10 mM/l of glucose, and 0.5 g/l of sodium thioglycollate.

By applying standard statistical calculations to the data shown in Table 3 the two ratios in Experiment D and similarly those in Experiment E are shown to differ from each other by more than three times standard deviation, and therefore the germination treatment is shown to result (at the 5.0% level of probability) in increased spore inactivation when compared with the inactivation of spores that were untreated but protected by a small quantity of sodium thioglycollate.

The germination inducing treatment of anaerobic spores appears to have little prospect of practical application as a radiation sensitizing agent unless it can be discovered how germination may be induced without the presence of sodium thioglycollate or some other substance that protects spores against radiations.

Observations on Bacillus cereus. To avoid the use of sodium thioglycollate in these spore germination experiments and to verify the findings reported previously, it was decided that an aerobic species should be studied. *Bacillus cereus* var. *terminalis* was chosen as the subject species because its germination requirements had previously been established *(11)*. In these experiments the germination medium consisted of 100 mM of L-alanine and one drop of a saturated water solution of adenosine per 3 ml. of medium. The heat sensitization of these spores was not so great as that observed in the experiments with spores of P. A. No. 3679 but was well defined.

An experiment (F) with irradiation of treated spores revealed that the extent of the radiosensitization was far in excess of what would be expected on the basis of the heat sensitization data. It was further noted that treatment with 100 mM of L-alanine produced a more pronounced radiosensitization effect than did treatment with 1000 mM of L-alanine (Table 4). In either case treatment with L-alanine resulted in an increased spore inactivation beyond that to be expected on the basis of the thermolability determinations and the normal radiosensitivity of these spores. The treatment with L-alanine, in addition to producing germination of a proportion of the spores, also produced radiosensitivity in another group of spores that had retained their heat resistance. This phenomenon was confirmed by Experiment G (Table 4).

Additional experiments were performed (Tables 5 and 6) to test the effects of treatment with 100 mM of L-alanine and adenosine and treatment with adenosine alone. A concentration of 100 mM of L-alanine caused the greatest sensitization effect of all the treatments tested. Adenosine alone was more effective in promoting spore radiosensitivity than was 1000 mM of L-alanine combined with adenosine. Comparison of the results obtained after treatment with adenosine alone and after treatment with L-alanine indicates that the addition of L-alanine in high concentration appeared to protect the spores. All treatments produced a radiosensitization greater than was expected on the basis of thermolability determinations.

SUMMARY

Experiments are reported indicating that the induction of ultra-rapid germination of bacterial spores of P. A. No. 3679 and *Bacillus cereus* by amino acids can increase the radiosensitivities of these organisms.

The presence of sodium thioglycollate in the germination medium was

shown to interefere with the demonstration of this effect on spores of P. A. No. 3679.

Bacillus cereus spores were radiosensitized by L-alanine and adenosine to an extent beyond that expected on the basis of thermolability determinations.

TABLE 4
Radiation sensitization[1] of Bacillus cereus spores

Experiment and sample	Dilution factor	No. of plates	Mean viable count	Standard deviation	Mean survivors (%)
Experiment F					
Untreated	10^6	4	58.5	3.95	.....
Untreated; repasteurized	10^7	4	73.5	7.83	.....
Untreated; irradiated	10^4	8	31	7.96	5.3
Treated with 100 mm/1 alanine	10^6	4	41.3	3.84	.....
Treated with 100 mm/1 alanine; repasteurized	10^6	4	36.3	6.50	87.7
Treated with 100 mm/1 alanine; irradiated	10^3	8	17.6	4.77	0.04
Treated with 1000 mm/1 alanine	10^6	4	60	7.04	.....
Treated with 1000 mm/1 alanine; repasteurized	10^4	4	130	9.67	23.3
Treated with 1000 mm/1 alanine, irradiated	10^3	8	77.6	24.9	0.13
Experiment G					
Untreated	10^6	8	39.7	5.36	.....
Untreated; repasteurized	10^6	8	38.2	5.58	96.3
Untreated; irradiated	10^4	8	31.9	7.36	8.0
Treated with 100 mm/1 alanine	10^6	8	32.8	3.39	.....
Treated with 100 mm/1 alanine; repasteurized	10^4	8	114	6.7	34.8
Treated with 100 mm/1 alanine; irradiated	10^3	8	41	5.85	0.01
Treated with 1000 mm/1 alanine	10^6	8	33	6.37	.....
Treated with 1000 mm/1 alanine; repasteurized	10^4	8	185	7.62	56.0
Treated with 1000 mm/1 alanine; irradiated	10^3	8	62	7.14	1.9

[1] Irradiation dose: 600,000 rep.

[2] The germination medium for treated samples of one drop of a saturated water solution of adenosine and 100 or 1000 mm/1 of L-alanine, as indicated in column 1.

TABLE 5

Radiation sensitization[1] of Bacillus cereus spores by treatment with a mixture of alanine plus adenosine[2] or by treatment with adenosine alone

Experiment and sample	Dilution factor	No. of plates	Mean viable count	Standard deviation	Mean survivors (%)
Experiment H					
Treated with adenosine	10^5	8	31.8	2.48	.....
Treated with adenosine; repasteurized	10^3	8	91.4	3.11	2.9
Treated with adenosine; irradiated	10^3	8	1	1.06	0.03
Treated with 10 mm/l alanine plus adenosine	10^5	8	33.6	3.18	.....
Treated with 10 mm/l alanine plus adenosine; repasteurized	10^4	8	156	6.32	46.5
Treated with 10 mm/l alanine plus adenosine; irradiated	10^3	8	17	1.90	0.005
Treated with 1000 mm/l alanine plus adenosine	10^5	8	33	2.73	.....
Treated with 1000 mm/l alanine plus adenosine; repasteurized	10^4	8	128	3.34	39.0
Treated with 1000 mm/l alanine plus adenosine; repasteurized	10^3	8	5.5	1.58	0.17

[1] Irradiation dose: 600,000 rep.
[2] One drop of a saturated water solution of adenosine.

TABLE 6

Radiation sensitization[1] of Bacillus cereus spores by treatment with a mixture of alanine plus adenosine[2] or by treatment with adenosine alone

Experiment and sample	Dilution factor	No. of plates	Mean viable count	Standard deviation	Mean survivors (%)
Experiment I					
Untreated	10^6	8	25	2.45	.....
Untreated; repasteurized	10^6	8	27	4.14	.....
Untreated; irradiated	10^4	8	26	2.60	10.4
Treated with adenosine	10^6	8	27	3.95	.....
Treated with adenosine; repasteurized	10^6	8	71	4.56	2.6
Treated with adenosine; irradiated	10^3	8	11	3.97	0.04
Treated with 10 mm/l alanine + adenosine	10^6	8	28	5.30	.....
Treated with 10 mm/l alanine + adenosine; repasteurized	10^4	8	2	0.79	0.71
Treated with 10 mm/l alanine + adenosine; irradiated	10^3	8	30	3.04	0.011
Treated with 1000 mm/l alanine + adenosine	10^6	8	24	1.84	.....
Treated with 1000 mm/l alanine + adenosine; repasteurized	10^4	8	4	0.93	1.7
Treated with 1000 mm/l alanine + adenosine; irradiated	10^2	8	62	2.30	0.26

[1] Irradiation dose: 600,000 rep.
[2] One drop of a saturated water solution of adenosine.

LITERATURE CITED

1. ANDERSEN, A. A. A rapid plate method of counting spores of *Clostridium botulinum*. *J. Bacteriol.*, **62**, 425-432 (1951).
2. CHURCH, B. D., HALVORSEN, H., AND HALVORSEN, H. O. Studies on spore germination: Its independence from alanine racemase activity. *J. Bacteriol.*, **68**, 393-399 (1954).
3. HACHISUKA, Y., ASANO, N. KATO, N., AND KUNTO, T. Decrease of optical density of spore suspensions *(B. subtilis)* and its relation to spore germination. *Nahoya J. Med. Sci.*, **17**, 403-410 (1954).
4. HALVORSEN, H. O. Factors determining survival and development of *Clostridium botulium* in semi-preserved meats. *Ixier Symposium Internat. de Bacteriol. Aliment.*, **7**, 53-67 (1955). (Annales de l'Institut Pasteur de Lille).
5. HAYNER, J. H., AND PROCTOR, B. E. Investigations relating to the possible use of atomic fission products for food sterilization. *Food Technol.*, **7**, 6-10 (1953).
6. HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes. The effect of yeast extract on the germination of *Bacillus anthracis* and its replacement by adenosine. *Biochem. J.*, **45**, 353-362 (1949a).
7. HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes. The effects of amino acids on the germination of *Bacillus anthracis*, with some observations on the relation of optical form to biological activity. *Biochem. J.*, **45**, 363-370 (1949b).
8. HILLS, G. M. Chemical factors in the germination of spore-bearing aerobes: Observations on the influence of species, strain and conditions of growth. *J. Gen. Microbiol.*, **4**, 38- 47 (1950).
9. HITZMAN, D., ZOHA, S. M. S., AND HALVORSEN, H. O. The requirements for germination of spores of some anaerobic bacteria. *Bact. Proc.*, p. 40 (1955).
10. POWELL, J. F. Factors affecting the germination of thick suspensions of *Bacillus subtilis* spores in L-alanine solution. *J. Gen. Microbiol.*, **4**, 330 (1950).
11. STEWART, B. T., AND HALVORSEN, H. O. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. *J. Bacteriol.*, **65**, 160-166 (1953).

SOME FACTORS INFLUENCING THERMAL RESISTANCE VALUES OBTAINED BY THE THERMORESISTOMETER METHOD*

J. L. SECRIST AND C. R. STUMBO

Food Research Department, H. J. Heinz Company, Pittsburgh 30, Pennsylvania

(Manuscript received May 14, 1957)

The importance in knowing the true nature of thermal death time curves lies in the fact that reliable curves are basic to the accurate calculation and evaluation of sterilizing processes. The straight-line semi-logarithmic nature of curves representing resistance of bacterial spores to temperatures up to 270° F. (132° C.) has been quite well established *(3, 8)*. However, there has been some doubt expressed recently *(3)* as to whether the curves continue into a higher temperature zone (270° F. to 300° F.) (132° C. to 149° C.) as straight lines. The reason postulated to explain the deviations noted was that the higher temperatures operating for short periods of time may not elicit chemical changes in the foods, which influence resistance of spores suspended therein, to the same extent as lower temperatures operating over longer periods of time. The present study was undertaken primarily because the above explanation appeared untenable in view of the results reported and because it was felt that the thermoresistometer method had been applied beyond its limitations of precision.

The explanation appeared untenable because a similar deviation from a straight line was reported for spores suspended in distilled water. It is difficult to understand how deviations in this case could be due to high-temperature or low-temperature induced chemical changes in the suspending medium. Also, no corrections for heating and cooling lags were made, yet errors due to these lags should have been appreciable for the extremely short heating times employed. Furthermore, appropriate corrections would definitely have tended to bring the points into straight lines.

It is assumed that the reason corrections were not applied was because in an earlier piece of work *(5)* it was impossible to show experimentally that corrections would significantly influence the results. For this reason the hazards of using phosphate buffers as suspending media in the thermoresistometer method have been explored in this study. It appeared possible that the erratic nature of data reported *(5)*, on which the reason for not making corrections must have been based, was due to variable evaporation from phosphate buffer samples prior to exposing them to lethal temperatures.

METHODS

All procedures involved in media preparation, in spore production and in estimation of spore concentrations were essentially the same as those described elsewhere *(8)*.

A liver broth medium was employed for production of the spores and the thermoresistometer method *(7)* was used for destroying vegetative cells and activating spores

* Presented at the Seventeenth Annual Meeting of the Institute of Food Technologists, Pittsburgh, Pennsylvania, May 14, 1957.

in connection with spore enumeration. Corrections were applied to heating times (U) at each of the various temperatures to compensate for the time required at 220° F. (104° C.) (0.464 minutes) to activate the spores of PA 3679. Pork infusion agar was employed throughout this study for subculturing heated spores.

Counts were made of spores activated in each of the media in which the spores were to be suspended for heat resistance determinations. Since no differences in spore counts were noted due to the nature of the suspending medium, all counts were averaged to obtain the number used in calculating D values. For establishing the nature of thermal death time curves, thermal resistance of PA 3679 spores in strained peas and distilled water was determined at 250° F. (121° C.), 260° F. (127° C.), 270° F. (132° C.), 280° F. (138° C.), and 290° F. (143° C.). In studies involving different concentrations of phosphate to make neutral buffer solutions and in studies to determine the effect of sample drying, resistance was determined at 240° F. (116° C.) and 260° F. (127° C.).

Preparation, inoculation and heating of all samples were as described elsewhere *(8)*. Each sample was inoculated with approximately 10,000 spores, and 6 replicate samples were subjected to each time-temperature relationship used. Results of subculture and incubation were evaluated as previously described *(8)*. All subcultures were incubated for 6 weeks at 85° F. to 90° F. (29° C. to 32° C.).

The inoculated suspending medium was chilled to approximately 39.2° F. (4° C.) and pipetted into sample cups pre-chilled to the same temperature. Immediately after inoculation, the samples were refrigerated again at 39.2° F. (4° C) until they were placed in the pre-exposure chamber of the thermoresistometer.

All phosphate buffer solutions were prepared according to the method of Sörensen *(2)*. Equi-molecular solutions of Na_2HPO_4 and KH_2PO_4 were mixed to give solutions of pH 7.0.

Initial temperatures of cup samples were determined for each exposure temperature. Values of I [exposure temperature (T_2) minus initial temperature (T_1)] appear in Table 1. These values were used in the calculation of t_c (lag correction) at each exposure temperature.

The desired exposure temperature (T_2) was first set in the exposure chamber. Then the cups were placed in the carrier plate inside the pre-exposure chamber. The temperature of the contents of the first cup placed was measured during the period of normal six-cup loading. The value of T_1 was recorded after the last cup was placed and the samples were ready to be moved into the exposure chamber. The temperature of the samples were recorded using an Ecklund thermocouple connected to a Brown electronic recording potentiometer. It was noticed that after the initial surge (less than 5 seconds) of temperature rise, the temperature of the samples remained constant within ± 1.0° F.

TABLE 1

Temperatures of thermoresistometer cup samples[1]

Exposure Temperature (T_2)	Initial Temperature (T_1)	I (approx.) ($T_2 - T_1$)
240°9.	174°F.	65°F.
250°F.	182°F.	70°F.
260°F.	186°F.	75°F.
270°F.	188°F.	80°F.
280°F.	193°F.	85°F.
290°F.	199°F.	90°F.

[1] 0.02 ml. samples of 1 : 1 Strained Pea-Distilled Water mixture.

TREATMENT OF DATA

The basic mathematical procedures are similar to those previously reported *(5, 8)*. It is understandable that a lag correction factor (t_c) should be applied to thermal

death time data in the temperature range 290° F. (143° C.) and above. A small correction in temperature lag may well alter the true value of U (heating time) when this time is necessarily short to begin with.

The equation for t_e is:

$$t_e = B_s - U \qquad (5)$$

where:

B_s = total heating time

U = exposure time, assuming instantaneous attainment of test temperature and instantaneous cooling after heating U minutes.

The heated samples are treated as infinite slabs. It is assumed that the samples heat by conduction and that the top and bottom surfaces of the samples approach the exposure temperature the instant the samples are drawn into the exposure chamber.

Under these conditions, assuming all temperatures within 0.1° of test temperature are at test temperature:

g = 0.1 (the sample reaches a temperature of 0.1° F. below exposure temperature)

j = 1.273 (4)

The formula for the f_h of the sample is:

$$f_h = 0.933 \frac{S^2}{K}$$

where S = sample half thickness

K = thermal diffusivity constant.

The diameter of the thermal death time cup measures 0.9 cms. and a 0.02 ml. volume of sample is spread uniformly over the inner bottom surface of each cup.

Since: $V = \pi r^2 h$

Then: $h = \frac{0.02 \text{ cm}^3}{(3.146)(0.45 \text{ cm})^2} = 0.0315 \text{ cm} = 0.0124 \text{ inches}$

and: $S = 0.5\ h = 0.0062$ inches

The diffusivity constant (K) for strained peas was determined using 211 x 400 containers with internal dimensions of 2.564 inches in diameter and 3.628 inches in length.

The equation for K (4) follows:

$$K = \frac{0.398}{(1/a^2 + \frac{0.427}{b^2})\ f_h}$$

a = ½ diameter = 1.282 inches

b = ½ length = 1.814 inches

An f_h value of 40.5 was obtained utilizing newer concepts of f_h correction factors due to thermocouple error *(6)*.

Substituting in the above equation and solving for K, we obtain a value of 0.0133 square inch per minute for strained peas. Then for 0.02 ml. samples in thermoresistometer cups:

$$f_h = 0.933 \frac{s^2}{K}$$

$$f_h = 0.933 \frac{(.0062)^2}{0.0133}$$

$$f_h = 0.0027$$

Now, according to Ball *(1)*:

$$B_s = f_h (\log j\ I - \log g)$$

Assuming the z value of the test bacterial spore culture is 18, then

$$f_h/U = 0.58 \text{ or } U = 1.725\ f_h$$

By substitution, the value of t_e becomes

$$t_e = f_h (\log j\ I - \log 0.1) - 1.725\ f_h$$

Using experimental procedures described above, a value of I ($T_s - T_i$) of 90° F. (32° C.) was obtained when the exposure temperature (T_s) was 290° F. (143° C.).

The value of j I then is equal to

$$1.273 \times 90 = 115$$

and the t_e value at 290° F. (143° C.) is:

$$t_e = f_h (\log 115 - \log 0.1) - 1.725\, f_h$$

$$t_e = f_h (3.061 - 1.725)$$

$$t_e = (0.0027)(1.336)$$

$$t_e = 0.0036 \text{ minutes}$$

The corrected value of U is:

$$U = B_B - t_e$$

and the D_{250} value is:

$$D_{250} = \frac{U \text{ (corrected)}}{\log a - \log b}$$

EXPERIMENTAL AND RESULTS

Initial studies were designed to determine if heating and cooling lags in samples subjected to heat for very short times in the thermoresistometer were of such magnitude as to cause serious errors if ignored—that is, considered negligible. Without correcting lags, D values were determined for PA 3679 spores suspended in strained peas and in distilled water. Temperatures employed were 250° F. (121° C.), 260° F. (127° C.), 270° F. (132° C.), 280° F. (138° C.), and 290° F. (143° C.). For each trial, 6 replicate samples were subjected to each time-temperature relationship. Each trial was repeated 8 times in strained peas and 4 times in distilled water making a total of 48 and 24 samples, respectively, subjected to each time-temperature relationship. Average D values calculated from the results appear in Table 2. Thermal death time curves constructed with the D values appear in Figures 1 and 2.

It may be noted that both curves are straight through 250, 260, 270 and 280 degree points. The 290 degree point, when no correction was made for lags, lies above the straight line through the other points in each case. Since this occurred with both strained peas and distilled water, it seemed unlikely that it could be a chemical change in the suspending medium affecting thermal resistance, as postulated by Esselen and Pflug *(3)*. Correcting for heating and cooling lags as outlined above brings, in the case of strained peas, the 290 degree point almost in line—the 280 degree point was shifted only very slightly by lag corrections. Correcting for lags, in the case of distilled water, moved the 290 degree point to slightly below the straight line.

It is recognized that corrections based on lags at the geometrical centers of samples will be somewhat greater than they should be. To ascertain the true correction would require integration of lethal effects throughout the sample—there is no method available for doing this for a sample that is classified as an infinite slab. Realizing that we are dealing with total heating times [at 290° F. (143° C.)] of only a few hundredths of a minute in length, we feel that it would be presumptuous to believe heating and cooling lags are the only factors which contribute errors to the results. Further, though we refer to heating time at 290° F. (143° C), let us determine for how much of the total heating time the center of the sample is at 290° F. (143° C.).

$$B_B = f_h (\log j\, I - \log g)$$

Assume g of 0.1 or maximum temperature reached 189.9, then from values under "Treatment of Data" above:

$$B_B = 0.0027 \text{ (log 115 — log 0.1)}$$
$$B_B = 0.00825 \text{ minutes}$$

The average total heating time for the samples of strained peas heated at 290° F. (143° C.) was 0.035 minutes, and for samples of distilled water, 0.021 minutes. Obviously, a great deal of the lethal effect at the center of the sample was by temperatures below 290° F. (143° C.).

In view of these considerations, to obtain an exact value to represent thermal resistance, when heating times must be as short as those used in this study to obtain 290 degree values, would appear scarcely short of fortuitous. Certainly, we cannot justifiably consider deviations such as those pictured in Figures 1 and 2 as indicating curvature of the thermal death time curves until some method is available to make more accurate determinations. The accuracy requirement is beyond the limit of the thermoresistometer method

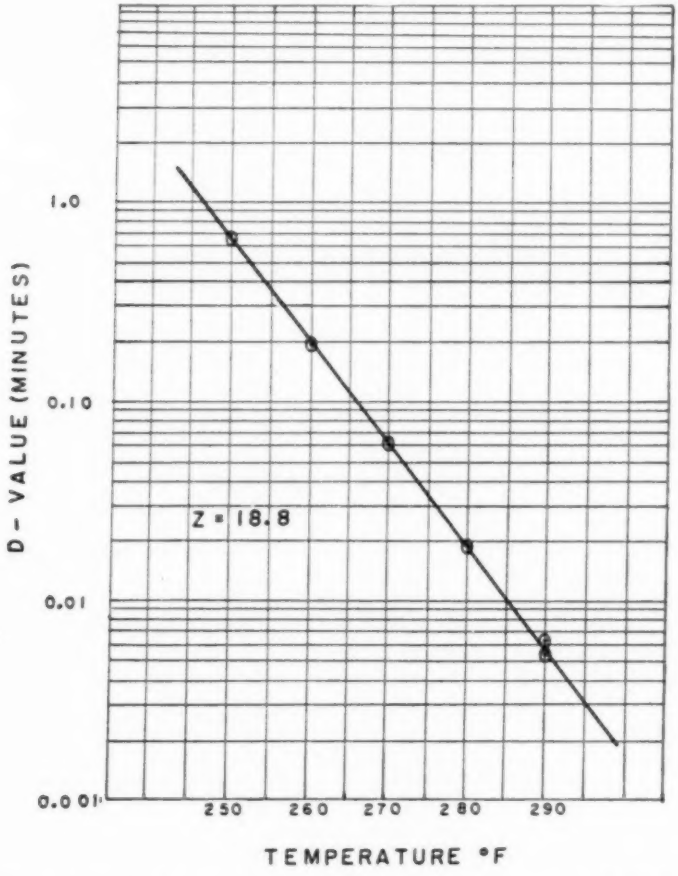


Figure 1. Thermal death time curve for NCA PA 3679 spores in distilled water.

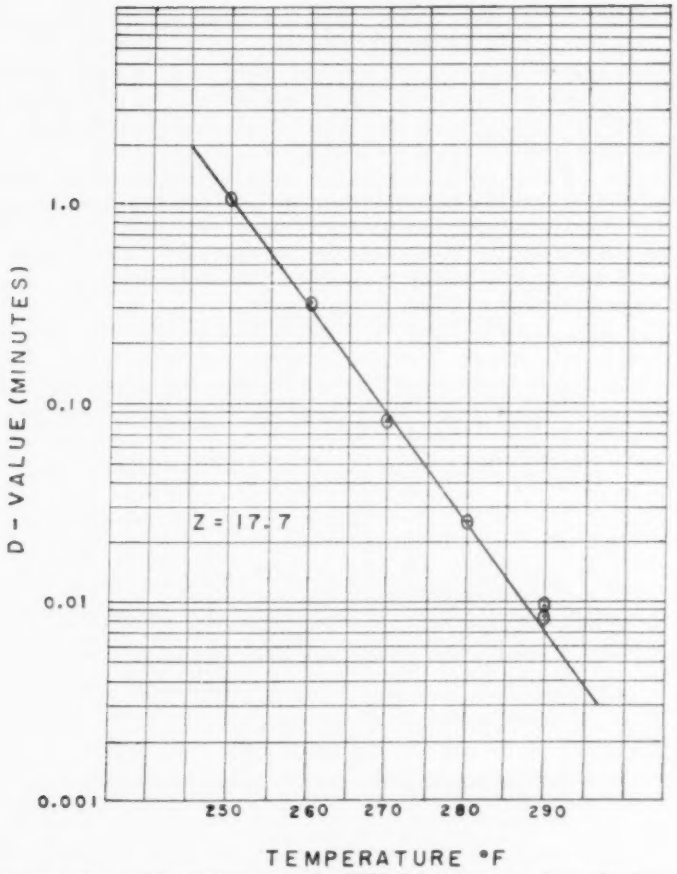


Figure 2. Thermal death time curve for NCA PA 3679 spores in strained peas.

and only rough approximations should be expected when using the method under such circumstances.

Soon after development of the thermoresistometer, a limited study (unpublished) was carried out to determine the effect on thermal resistance of evaporation from phosphate buffer solutions when they were used as spore suspending media. The chances for considerable evaporation from the sample cups after they are placed in the carrier plate of the thermoresistometer are certain if there is any appreciable delay between placing the samples in the plate and drawing them into the exposure chamber. Results of the study showed that evaporation caused an increase in spore resistance, and since the extent of evaporation was often variable among samples, it was usual to observe very erratic results, particularly in the form of skips. No such effect was observed for a number of different food products used as suspending media.

TABLE 2

D values for NCA PA 3679 spores in strained peas and distilled water.

Exposure Temperature	D Values			
	Uncorrected		Corrected	
	Strained Peas	Distilled Water	Strained Peas	Distilled Water
250°F.	1.120	0.726		
	0.946	0.652		
	1.060	0.668		
	0.980	0.634	Corrected Values same to 3rd Decimal Place	
	1.250			
	1.140			
	1.080			
	1.120			
Average	1.087	0.670		
260°F.	0.329	0.195		
	0.325	0.207		
	0.282	0.201		
	0.300	0.187	Corrected Values same to 3rd Decimal Place	
	0.352			
	0.321			
	0.308			
	0.308			
Average	0.316	0.198		
270°F.	0.080	0.060		
	0.083	0.062		
	0.084	0.060		
	0.073	0.065	Corrected Values same to 3rd Decimal Place	
	0.088			
	0.080			
	0.077			
	0.084			
Average	0.081	0.062		
280°F.	0.023	0.018	0.022	Corrected Values same to 3rd Decimal Place
	0.024	0.021	0.023	
	0.025	0.018	0.024	
	0.028	0.020	0.027	
	0.026		0.025	
	0.025		0.024	
	0.026		0.025	
	0.024		0.024	
Average	0.025	0.019	0.024	
290°F.	0.0099	0.0062	0.0091	0.0053
	0.0099	0.0063	0.0090	0.0054
	0.0084	0.0068	0.0076	0.0059
	0.0082	0.0059	0.0073	0.0049
	0.0095		0.0086	
	0.0095		0.0086	
	0.0094		0.0085	
Average	0.0093	0.0063	0.0084	0.0054

The more detailed study reported here confirms these earlier observations. Four different concentrations of phosphate buffer, prepared as described above under "Methods," and distilled water were employed as suspending media to determine the effect of phosphate concentration on thermal resistance of PA 3679 spores. All inoculated samples were heated at 260° F. (127° C.)

and replicates ranged from 18 to 24. All samples after preparation were refrigerated until just prior to placing them in the thermoresistometer, and drawn into the exposure chamber as soon as possible thereafter. Results appear in Table 3. Though the increase in resistance with increase in phosphate concentration appeared obvious, the data were subjected to statistical evaluation. The difference in resistance of the spores in distilled water and in M/15 phosphate buffer was found to be highly significant. Likewise, the differences in resistance of the spores in M/15 buffer and in M/3.75 and M/1.875 buffer were found to be highly significant.

Further studies demonstrated that evaporation from phosphate buffer samples in the carrier plate of the thermoresistometer, when the samples were allowed to remain in the plate for extended periods of time before being drawn into the exposure chamber, caused similar increases in resistance. However, unless evaporation among samples was well controlled, resistance values observed were quite variable. We found it very difficult to control the evaporation when the thermoresistometer was being operated at temperatures above 240° F. (116° C.). But, the erratic nature of such results further

TABLE 3

Thermal resistance of NCA PA 3679 spores in phosphate buffers

Suspending Medium	pH	D_{240} Values	Difference
Distilled Water	7.1	0.204	Very Highly Significant .001
M/15 Phosphate Buffer	7.0	0.382	Not Quite Significant at .05
M/7.5 Phosphate Buffer	7.0	0.454	Highly Significant .01
M/3.75 Phosphate Buffer	7.0	0.513	Not Quite Significant at .05
M/1.875 Phosphate Buffer	7.0	0.564	

supports the conclusion that when using phosphate buffer as suspending medium for spores to be heated in the thermoresistometer, extreme caution must be exercised to keep evaporation from samples to a minimum prior to their being heated.

Results in Tables 4 and 5 show the effect of evaporation in samples heated at 240° F. (116° C.) after they had been allowed to remain in the carrier

TABLE 4

Thermal resistance of NCA PA 3679 spores in M/15 Phosphate Buffer subjected to drying

Drying Time (Minutes)	D_{240} Values
Zero	5.84
1.5	8.32
3.0	9.39

TABLE 5
Thermal Resistance of NCA PA 3679 spores in strained peas subjected to drying

Drying Time (Minutes)	D_{250} Values
Zero	4.25
1.5	4.91
3.0	5.19

plate for 0.00, 1.50, or 3.00 minutes prior to their being drawn into the exposure chamber. In this study 240° F. (116° C.) was purposely chosen because the carrier plate was not so hot as to preclude reasonably accurate timing of the preheating periods. Statistical evaluation of the data shows the differences between the D values for spores in strained peas preheated for the different lengths of time not to be significant at the 5% level. Differences in D values for the spores in M/15 phosphate buffer preheated for the different lengths of time were significant.

SUMMARY

Thermal resistance values are presented for PA 3679 spores suspended in strained peas and distilled water and subjected to temperatures in the range 240° F. (116° C.) to 290° F. (143° C.). Thermal death time curves constructed from these data are straight lines through the point representing 280° F. (138° C.), without corrections for heating and cooling lags. The point representing 290° F. (143° C.) lies above the straight line in each case. Approximating a correction for heating and cooling lags and applying it tends to bring the 290° point into line. Reasons for expecting deviations when heating times are very short are discussed. It is concluded that methods for more accurate measurement of thermal resistance, when heating times are extremely short, will have to be developed before the true nature of thermal death time curves for the higher temperature range, above 280° F. (138° C.) in the case of PA 3679 spores, can be determined. There is no indication from data presented here that true curves are not straight lines.

Data showing the effect of phosphate concentration in neutral buffer solutions on thermal resistance of spores suspended in the solutions are presented. Thermal resistance was found to increase with increases in phosphate concentration. It was also found that if evaporation of phosphate buffer samples were allowed in the pre-exposure chamber of the thermoresistometer, erratic results could be expected, for reasons of increasing the phosphate concentration of the suspending medium. This effect of evaporation (concentration of sample) on thermal resistance was not observed for strained peas. Previous work had indicated it was not important for a number of other food products and for distilled water.

Acknowledgment

The authors wish to acknowledge the cooperation and assistance of Mr. I. MacPhail, Quality Control Department, H. J. Heinz Company, for his statistical treatment of the data.

LITERATURE CITED

1. BALL, C. O. Thermal process time for canned foods. *Bull.* Nat'l Research Council, **7**, Part 1, No. 37 (1923).
2. CLARK, W. M. *The determination of Hydrogen Ions.* 2nd ed., 1925. Williams and Wilkins Co., Baltimore, Maryland.
3. ESSELEN, W. B., AND PFLUG, I. J. Thermal resistance of putrefactive anaerobe No. 3679 spores in vegetables in the temperature range of 250-290° F. *Food Technol.*, **10**, 557 (1956).
4. OLSON, F. C. W., AND JACKSON, J. M. Heat curves. Theory and practical application. *Ind. Eng. Chem.*, **34**, 337 (1942).
5. PFLUG, I. J., AND ESSELEN, W. B. Heat transfer into open metal thermoresistometer cups. *Food Research*, **20**, 237 (1955).
6. SEGMILLER, J. L., AND STUMBO, C. R. Heat conduction through thermocouple wire a factor in heat penetration measurement. Paper No. 121 presented before the 17th Annual Meeting of the Institute of Food Technologists, Pittsburgh, Pa. (1957).
7. STUMBO, C. R. A technique for studying resistance of bacterial spores to temperatures in the higher range. *Food Technol.*, **2**, 228 (1948).
8. STUMBO, C. R., MURPHY, J. R., AND COCHRAN, J. Nature of thermal death time curves for PA 3679 and *Clostridium botulinum*. *Food Technol.*, **4**, 321 (1950).

BROWNING OF SUGAR SOLUTIONS

I. Effect of pH and Type of Amino Acid in Dilute Sugar Solutions[a]

C. O. WILLITS, J. C. UNDERWOOD, H. G. LENTO, JR.,[b] AND C. RICCIUTI

Eastern Regional Research Laboratory,[c] Philadelphia 18, Pennsylvania

(Manuscript received May 14, 1957)

Many years of research on the browning of food materials has shown that, under favorable conditions for each, many organic compounds react in various ways to form dark-colored products. After Maillard *(5)* published his classical work on the reaction of amino and carbonyl groups, most of the work on browning was directed toward finding the mechanism of the formation of the colored reaction products of amino compounds and reducing sugars, stressing the role of the amino compounds. That little study has been directed toward other reactants of the browning mechanism is indicated by excellent reviews of the subject which have been published by Hodge *(2)* and Liggett and Deitz *(4)*. More recently, however, Schroeder, Iacobellis and Smith *(6)* have proposed the theory that browning and the Maillard reaction may occur independently of each other. They found that browning (expressed in qualitative terms) in glucose solutions appeared to be largely due to the effect of pH on the hexose, and not to the interaction between amino compounds and carbohydrates. Taufel and Iwainsky *(7)* have reported similar results. Joslyn *(3)* has shown that the amino acids play a minor role in browning.

Since the typical brown color of maple sirup is produced in the presence of almost no amino compounds (0.001% *N* in maple sap), a type of nonamino browning is indicated. Therefore, studies at this Laboratory on the control of color and on the development of flavor in maple sirup have been directed primarily toward the sugars, which are the major constituents of maple sap and sirup. It is quite possible that alkaline degradation of the hexoses in the sap is part of the mechanism of color development in maple sirup, since the sap passes through a stage of alkaline pH, during the evaporation *(1)*. As to the amino-carbonyl reaction, it is well known that this occurs best in concentrated solutions or in the dry state, whereas the browning of maple sirup takes place in a dilute solution.

In an effort to clarify the browning mechanism as it applies to maple sap, the authors have for some time been conducting experiments with model systems of dilute glucose solutions both with and without amino acids and under a variety of pH conditions. This paper presents the results of some of these studies on the effect of pH and type of amino acid on the browning of dilute glucose solutions. Statistical analysis of the data provide further evidence to substantiate the theory that the effect of pH on the hexoses in the sap are primarily responsible for the browning reaction in maple sirup.

---

[a] Presented at the Seventeenth Annual Meeting of IFT, Pittsburgh, Pa., May 14, 1957.

[b] From work done for a thesis to be submitted by H. G. Lento to the Graduate School of Georgetown University as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[c] One of the laboratories of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

EXPERIMENTAL

Effect of pH and individual amino acids in glucose solutions. To a series of 48 portions of 10 ml. of distilled water in 50-ml. beakers was added 1 ml. of a 1.25 *M*. glucose solution. The series was divided into 4 sets of 6 solutions each in duplicate. To each of the portions in 3 sets, 5 ml. of 1.25 *M*. solutions of L-lysine monohydrochloride, DL-alanine, and L-glutamic acid monohydrochloride were added respectively. The fourth set, the control, contained only the glucose. The solutions in each of the 4 sets were adjusted to the desired pH (3, 5, 6, 7, 8, and 9 ± 0.05) by titration with either 1 *N*. sodium hydroxide or 1 *N*. phosphoric acid. Immediately 15 ml. of the appropriate phosphate buffer was added to maintain the desired pH constant throughout the heating period. The buffered solutions were quantitatively transferred to 50-ml. volumetric flasks and made to volume with distilled water. The solutions were 0.025 *M*. in respect to glucose and 0.125 *M*. in respect to the amino acid. Ten milliliters of each of these solutions were transferred to a 16 mm. x 15 cm. test tube (drawn down to 2 mm. 5 cm. from the open end of the tube). To fill the tubes they were first warmed and then the 10 ml. of solution was added to the top funnel-like portion. The tube was then chilled in ice water and the solution drawn into the lower part of the tube with 2 cm. of free space between the liquid and the constricted portion. The tubes were allowed to drain a few minutes, after which the constricted portion was sealed off with a flame.

The glucose solutions in the sealed tubes were heated at 114° C. by autoclaving at 10 p.s.i. for 20 minutes. Following this, the tubes were immediately chilled in ice water. The tubes were opened at the seal and aliquots removed for measurement of color.

TABLE 1

Effect of individual amino acids and pH on the color developed in a 0.025 *M*. glucose solution heated for 20 minutes at 114°C. in an autoclave

Components	pH	Absorbance 500 mμ
Glucose	3	0.00
	5	0.00
	6	0.00
	7	0.00
	8	0.18
	9	0.48
Glucose + DL-alanine	3	0.00
	5	0.00
	6	0.00
	7	0.14
	8	0.20
	9	0.33
Glucose + glutamic acid	3	0.00
	5	0.00
	6	0.00
	7	0.02
	8	0.12
	9	0.50
Glucose + lysine	3	0.00
	5	0.00
	6	0.16
	7	0.35
	8	0.65
	9	0.84

Absorbances were measured at 500 mμ with a Cary recording spectrophotometer[4] in a 1-cm. cell. Solutions with absorbances greater than 0.8 were diluted before measuring. The results are given in Table 1.

Effect of pH and different concentrations of mixtures of 3 amino acids in glucose solutions. To determine the effect of such multiple factors as pH, mixtures of amino acids, and different concentrations of them on heated dilute solutions of glucose, a statistically designed experiment was conducted. It consisted of a 3x2x4 factorial arrangement with 3 amino acids at 2 concentrations each and 4 different pH's. All solutions contained the same concentration of glucose, 0.025 *M*. The amino acids were DL-alanine, L-glutamic acid monohydrochloride, and L-lysine monohydrochloride. The design was so arranged that each solution contained the 3 acids with the concentration of the individual acids either 0.0025 *M*. or 0.125 *M*., and was buffered at a pH of either 4, 6, 7, or 9.

To reduce the number of samples required to be handled at one time, the design was set up in two parts, each having a factorial of 3x2x2. This was identical with the above 3x2x4 except that pH 4 and 6 were used in one arrangement and pH 7 and 9 in the other. These solutions were made up, heated, and the resultant color measured as in the previous experiment. The absorbances of the solutions relative to distilled water are shown in Table 2.

TABLE 2

Effect of mixtures of amino acids at various levels of concentration on the browning of a 0.025*M*. glucose solution at pH 4, 6, 7 and 9 heated for 20 minutes at 114°C. in an autoclave

Components[1]	Absorbance at 500 mμ			
	pH 4	pH 6	pH 7	pH 9
Glucose	0.00	0.00	0.08	0.26
$A_1Gl_1L_1$	0.00	0.01	0.09	0.29
$A_1Gl_1L_2$	0.00	0.06	0.48	0.93
$A_1Gl_2L_1$	0.00	0.02	0.08	0.23
$A_1Gl_2L_2$	0.00	0.06	0.54	0.97
$A_2Gl_1L_1$	0.00	0.02	0.09	0.23
$A_2Gl_1L_2$	0.00	0.08	0.52	1.20
$A_2Gl_2L_1$	0.00	0.02	0.10	0.28
$A_2Gl_2L_2$	0.00	0.06	0.62	1.1

[1] A_1 = DL-Alanine at 0.0025 *M*. level.
A_2 = DL-Alanine at 0.125 *M*. level.
Gl_1 = L-Glutamic acid monohydrochloride at 0.0025 *M*. level.
Gl_2 = L-Glutamic acid monohydrochloride at 0.125 *M*. level.
L_1 = L-Lysine monohydrochloride at 0.0025 *M*. level.
L_2 = L-Lysine monohydrochloride at 0.125 *M*. level.

RESULTS

Effect of individual amino acids and pH. The amount of color developed in the various glucose solutions is recorded in Table 1 and in Figure 1 as absorbance at 500 mμ. The compounds DL-alanine, an example of the neutral amino acids, and L-glutamic acid monohydrochloride, an example of the acidic amino acids, had no apparent effect on the amount of browning which took place irrespective of the pH of the solution. However, the lysine, an example of the basic amino acids, significantly influenced the amount of color formed. In the solutions containing only glucose no browning occurred below pH 7, but above this point color began to form in increasing amounts as the alkalinity of

[4] Mention of trade names does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

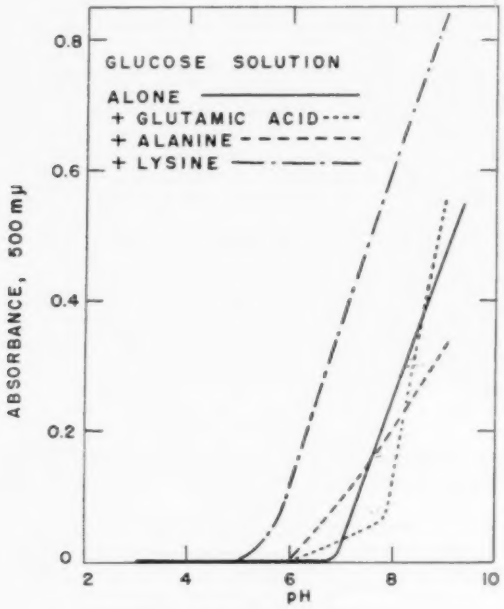


Figure 1. Absorbance at 500 mμ.

the solution increased. When lysine was present with the glucose at pH 7 and above, the solutions were browner than the corresponding controls. Further, this amino acid caused a small amount of color in the glucose solution at pH 6. This is in agreement with the observations made by Joslyn on the browning of orange juice *(3)*. Under the conditions of the present investigation those solutions containing either alanine or glutamic acid were not more colored than the controls; hence it was concluded that the degree of basicity of the solution was a dominant factor in the amount of color produced. This basic condition often occurs in natural products such as maple sirup, but it is a condition which has not been observed in the majority of the studies on browning which have emphasized the effect of pH upon the reaction of amino acids and reducing sugars.

Effect of mixtures of amino acids at different levels of concentration and pH. The data in Table 2 again show the necessity of an alkaline medium for significant color development in dilute solutions. Also, the positive effect of lysine was again demonstrated by the browning which occurred at pH 6, even at a very low level of concentration.

At pH 7 and pH 9 the reaction of the medium plus the effect of the amino acids caused the formation of significant color in all the treatments. Therefore, to determine the relative effects and whether or not there were interactions among the alanine, glutamic acid, lysine and pH, the data obtained by the 16 treatments (Table 2) were analyzed statistically.

The experimental design used was an unreplicated 2^4 factorial as shown in

Table 3. The data were treated by an analysis of variance and the main effects and the first-order interactions were tested using higher-order interactions as experimental error. The complete analysis is shown in Table 4. To attain significance at the 1% probability level, an F value of 16.26 was required. Therefore only the lysine x pH interaction is significant. It is also clear from this table that the effects of lysine and pH are unequivocally the only factors causing the formation of color in the glucose solution. However, because of the lysine x pH interaction these main effects have to be interpreted with caution. It will be helpful, therefore, to look at the table of means for these two factors (Table 5). It is apparent from this table that most of the treatment

TABLE 3

Experimental design of the 2^4 factorial showing combinations of the four factors pH, alanine, glutamic acid, and lysine

	A_1				A_2			
	Gl_1		Gl_2		Gl_1		Gl_2	
	L_1	L_2	L_1	L_2	L_1	L_2	L_1	L_2
pH 7	$7A_1Gl_1L_1$	$7A_1Gl_1L_2$	$7A_1Gl_2L_1$	$7A_1Gl_2L_2$	$7A_2Gl_1L_1$	$7A_2Gl_1L_2$	$7A_2Gl_2L_1$	$7A_2Gl_2L_2$
pH 9	$9A_1Gl_1L_1$	$9A_1Gl_1L_2$	$9A_1Gl_2L_1$	$9A_1Gl_2L_2$	$9A_2Gl_1L_1$	$9A_2Gl_1L_2$	$9A_2Gl_2L_1$	$9A_2Gl_2L_2$

A = Alanine
Gl = Glutamic acid
L = Lysine

Subscript 1 = low level of concentration
Subscript 2 = high level of concentration

TABLE 4

Results of statistical analysis

Source of Variation[1]	Degrees of Freedom	Sums of Squares	Mean Squares	F
Total	15	2.1868	.....	.....
A_1 vs. A_2	1	0.0105	0.0105	3.48
Gl_1 vs. Gl_2	1	0.0008	0.0008	0.27
L_1 vs. L_2	1	1.5563	1.5563	515.33
pH 7 vs. pH 9	1	0.4658	0.4658	154.24
A × Gl	1	0.0008	0.0008	0.27
A × L	1	0.0095	0.0095	3.15
A × pH	1	0.0033	0.0033	1.09
Gl × L	1	0.0011	0.0011	0.10
Gl × pH	1	0.0028	0.0028	0.93
L × pH	1	0.1208	0.1208	40.00
Error	5	0.0151	0.00302	.....
5% level	....	.....	.....	6.61[2]
1% level	....	.....	.....	16.26[2]

[1] Symbols same as in Table 3.
[2] Book value.

TABLE 5

Table of the means of lysine and pH Interaction

	L_1	L_2	Means
pH 7	0.09	0.54	0.32
pH 9	0.26	1.05	0.66
Means	0.18	0.80	

effects can be attributed to the synergistic effect of the lysine-pH complex when the solution is alkaline. There is, of course, definite indication of a pH effect in the absence of lysine and this confirms the data presented in Table 1. Also there was some indication of a lysine effect at pH 7.

All this suggests that a degradation of the sugar may be a primary reaction and a prerequisite in the browning mechanism. In these experiments browning occurred only in alkaline solutions except for those in which lysine was present. In these solutions a small amount of color was formed at a pH as low as 6. This could have been due to a local basicity effect of either one or both of the amino groups. This hypothesis was tested further by determining the effect on browning of two other basic amino acids, arginine and histidine. These, however, had no effect on the amount of browning in a 0.025 *M.* glucose solution. A further study of the increased color production due to the presence of lysine in an alkaline sugar solution will be reported in a subsequent paper.

The glucose solutions that were 0.0025 molar in respect to lysine (Table 2) contained 20 times the amount of nitrogen that is present in maple sap. Further, only a small percentage of the nitrogen in the sap is in the form of amino acids and a still smaller fraction is lysine. Therefore, the fact that no significant increase in color occurred in these solutions with the low-level concentration of amino acids, would be further evidence that amino acids are not responsible for the browning of maple sirup.

SUMMARY

The amount of color developed in a heated dilute glucose solution was influenced by the pH of the solution and type of added amino acid as follows:

1. Glucose solutions containing no amino acids developed color only when their alkalinity exceeded pH 7.
2. An alkaline medium was a prerequisite for significant browning in all cases.
3. The presence of alanine or glutamic acid in the glucose solution did not cause a significant increase in the amount of color produced relative to the control at any pH.
4. The glucose solutions containing lysine exhibited more browning at pH 7 and above than did similar solutions of only glucose. Further, this amino acid induced the formation of a small amount of color at pH 6.
5. The effect of lysine did not appear to be due to its basicity, since arginine and histidine, other basic amino acids, had no positive effect on browning.
6. Statistical analysis of the data shows that lysine and alkaline pH had a synergistic effect on the amount of color developed and that no other combination had such an interaction.

Extrapolation of these results on model systems to the browning of maple sirup indicates that amino acids have no part in this reaction.

Acknowledgments

The authors with to thank Morris D. Finkner, Biometrical Services, Agricultural Research Service, for his assistance in the statistical designing and analysis of the experimental work presented here, and to Anne M. Smith, Analytical Physical-Chemical and Physics Section of the Laboratory for the spectrophotometric measurements.

LITERATURE CITED

1. HAYWARD, F. W. AND PEDERSON, C. S. Some factors causing dark-colored maple sirup. N. Y. State Agr. Expt. Sta. *Bull. 718* (1946).
2. HODGE, J. E. Chemistry of browning reactions in model systems. *J. Agr. Food Chem.*, **1**, 928 (1953).
3. JOSLYN, M. A. The role of amino acids in the browning of orange juice. *Food Research*, **22**, 1 (1957).
4. LIGGETT, R. W., AND DEITZ, V. R. Color and turbidity of sugar products. *Advances in Carbohydrate Chemistry*, **9**, 247 (1954).
5. MAILLARD, L. C. Action of amino acids on sugars. Formation of melanoidin in a methodical way. *Compt. rend.*, **154**, 66 (1912).
6. SCHROEDER, L. J., IACOBELLIS, M. AND SMITH, A. H. The influence of water and pH on the reaction between amino compounds and carbohydrates. *J. Biol. Chem.*, **212**, 973 (1955).
7. TAUFEL, K. AND IWAINSKY, H. Analytical and chromatographic studies on the Maillard reaction. *Biochem. Z.*, **323**, 299 (1952).

BROWNING OF SUGAR SOLUTIONS
II. Effect of the Position of Amino Group in the Acid Molecule in Dilute Glucose Solutions[a]

H. G. LENTO, JR.,[b] J. C. UNDERWOOD, AND C. O. WILLITS
Eastern Regional Research Laboratory,[c] Philadelphia 18, Pennsylvania

(Manuscript received May 14, 1957)

In the previous paper of this series *(2)*, it was shown that when glutamic acid, alanine, or lysine was present in a dilute glucose solution heated at 114° C., only the lysine, a basic amino acid, caused a significant increase in browning. This effect of lysine became more pronounced with increasing alkalinity of the sugar solution. Acidic and neutral amino acids did not show this accelerating effect, nor did arginine and histidine, other basic amino acids. Therefore, since lysine, in addition to being basic, is a simple diamino compound it was postulated that the number and the position of the amino groups in the amino acid molecule might determine its activity in the browning mechanism. The purpose of the present investigation was to determine the effect on the color developed in a glucose solution at pH 8 of several series of amino acids containing one or two amino groups. A first series contained the 4-, 5-, and 6-carbon homologs of lysine with amino groups attached to the alpha and terminal carbons. A second and a third series were composed respectively of the 3-, 4-, 5-, and 6-carbon examples of the alpha-amino and the terminal amino monocarboxylic acids.

EXPERIMENTAL

Weighed amounts of each amino acid, equivalent to 10 ml. of a 0.1 molar solution, were placed in separate beakers containing 2 ml. of the phosphate buffer solution, made according to Hawk, Oser, and Summerson (1). The pH was adjusted to 8 by titration (Beckman glass electrode pH meter[d]) with 0.1 *N* NaOH. To stabilize and keep the pH constant during the heating an additional 3 cc. of the buffer was added, and the solution quantitatively transferred to a 10-ml. volumetric flask containing 1 ml. of a stock glucose solution (0.1801 g. glucose per ml.) and the solution made to volume. The resulting solutions were 0.1 molar in respect to both the particular amino acid and to the glucose. Five-milliliter aliquots of each of these solutions were heated in sealed tubes at 114° C., at 10 p.s.i., as described previously (*2*). After cooling, the absorbance of the browned solutions was measured at 500 mμ in a Cary recording spectrophotometer. Data presented in Table 1 are averages of duplicate determinations.

RESULTS AND DISCUSSION

The amount of browning varied widely as influenced by both the type and chain length of the amino acid present. Graphic presentation of the influence of the 3 different series of amino acids tested is shown in Figure 1. In general,

---

[a] Presented at the Seventeenth Annual Meeting of IFT, Pittsburgh, Pa., May 14, 1957.

[b] From work done for a thesis to be submitted by H. G. Lento to the Graduate School of Georgetown University as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

[c] One of the laboratories of the Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[d] Mention of trade names does not imply endorsement by the U. S. Department of Agriculture over similar products not mentioned.

TABLE 1

The effect of various amino acids on the color developed in a dilute glucose solution at pH 8

Amino acid	Formula	Number of carbon atoms	Terminal amino group	α Amino group	Absorbance 500 mμ
Glycine	NH_2CH_2COOH	2	+	+	1.56
DL-α-alanine	CH_3CHNH_2COOH	3	+	+	0.77
DL-β-alanine	$NH_2CH_2CH_2COOH$	3			2.00
DL-α-aminobutyric acid	$CH_3CH_2CHNH_2COOH$	4	+	+	1.00
DL-γ-aminobutyric acid	$NH_2CH_2(CH_2)_2COOH$	4	+		2.30
2,4-diaminobutyric acid.2 HCl	$NH_2CH_2CH_2CHNH_2COOH$	4	+	+	0.85
DL-norvaline	$CH_3(CH_2)_2CHNH_2COOH$	5	+	+	1.07
δ-aminovaleric acid.HCl	$NH_2CH_2(CH_2)_3COOH$	5	+		1.88
DL-ornithine.HCl	$NH_2CH_2(CH_2)_2CHNH_2COOH$	5	+	+	3.07
DL-norleucine	$CH_3(CH_2)_3CHNH_2COOH$	6	+	+	1.21
ε-aminocaproic acid	$NH_2CH_2(CH_2)_4COOH$	6	+		0.70
Lysine	$NH_2CH_2(CH_2)_3CHNH_2COOH$	6	+	+	4.10
Glucose	$CH_2OH(CHOH)_4CHO$				0.64

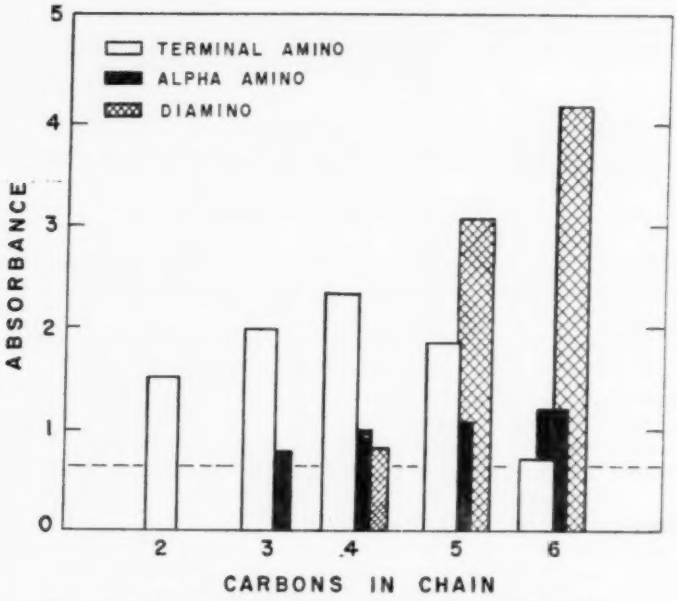


Figure 1. Influence on browning of three series of amino acids.

the monoamino acids containing the amino group in the alpha position caused only a small increase in color compared to those containing the amino group in the terminal position or to those containing two amino groups. The 6-carbon diamino acid, lysine, produced the greatest increase in color formation of all the 12 acids tested. This tremendous acceleration of browning by lysine does not seem to be wholly related to the general basicity which this compound would impart to a glucose solution. It was pointed out in the first paper of this series that arginine and histidine do not show the same positive effect as lysine. These two amino acids are basic in their reaction when in solution, but have different molecular structures than lysine. Another aliphatic diamino acid closely related to lysine, ornithine (δ,α-diamino valeric acid), also caused increased color development in the glucose solution. Apparently, then, the form of the molecular structure of the amino acid as well as its acid-base reaction in solution is a factor in determining its influence on browning.

The chain length of the amino acids produced an effect on the coloring of the glucose solution which varied with the type of amino acid. Figure 1 shows that the longer chained aliphatic alpha mono-amino acids, such as norvaline and norleucine, caused the production of only slightly more color than the shorter chained acids such as alanine. The absolute amounts of color which developed in the glucose solutions treated with monoamino aliphatic acids were only slightly more than the amount developed in the glucose control. Again it should be noted that the influence of the monoamino aliphatic acids as a whole was very slight compared to the terminal and diamino acids.

The monamino acids containing the amino group in the terminal position

produced variable results on browning. Increasing their chain length from 3 to 4 carbons caused a significant increase in the amount of color in the sugar solution. However, the 5-carbon acid, δ-amino valeric acid, produced a smaller amount of color than the 4-carbon acid. Further, the solution containing the 6-carbon acid, ε-amino caproic acid, did not show any more color than the glucose control. One possible explanation of this reversal in effect with increase in chain length is that the terminal amino group in 3- and 4-carbon monoamino acids are kept at a distance from the carboxyl group, whereas the amino group in 5-and in 6-carbon acids, because of the latter's ability to bend, is brought closer and closer to the carboxyl group, thus decreasing the effect of the amino group.

In the case of the diamino acids, the 2,4-diamino butyric acid caused only a small increase in color formation. However, the rate of increase in color formation with increase in chain length for this series was much greater than that for either of the two series of monoamino acids. Consequently the amount of color developed in all of the solutions tested was greater in the sugar solution containing lysine.

SUMMARY

In studying the effect of the positions of the amino groups in amino acids on the browning of glucose solutions it has been shown that the diamino acids caused the formation of the largest amount of additional color as compared to a glucose control. The greatest increase was caused by the 6-carbon diamino acid, lysine. The monoamino acids with the amino groups at the end of the chain caused varied changes in the color formation. Increased color formation occurred as the chain length of this series increased from 2 to 4 carbon atoms and then decreased as the chain length increased further to 5 and 6 carbon atoms. The monoamino acids with the amino group in the alpha position had almost no effect on the amount of color produced, regardless of chain length.

LITERATURE CITED

1. Hawk, P. B., Oser, B. L. and Summerson, W. H. *Practical Physiological Chemistry.* 12th ed., 1947. Blakistons & Sons, Philadelphia, Pa.
2. Willits, C. O., Lento, Jr., H. G., Underwood, J. C. and Ricciuti, C. Browning of sugar solutions. I. Effect of pH and type of amino acid. (In press).

A METHOD FOR THE CHARACTERIZATION OF PECTIC SUBSTANCES IN SOME FRUIT AND SUGAR-BEET MARCS

MILDRED GEE, ELIZABETH A. McCOMB, AND ROLLAND M. McCREADY
Western Regional Research Laboratory, Albany, Calif.*

(Manuscript received May 31, 1957)

Interest in the pectic substances has persisted because of their important role in maintaining the texture of fruits, vegetables, and their processed products *(3)*.

Previous researches, explaining the changes in pectic substances as related to texture, required the extraction of pectic substances, partial purification, and chemical characterization of the isolated material. Analyses include determination of percentage of esterification of uronic acid carboxyl groups, acetyl, anhydrouronic acid, gross solubility characteristics, and in some cases molecular weight *(9)*.

Pectic substances as high-molecular-weight carbohydrate polymers are intimately associated with the insoluble cellular materials in plants. Because of their association in the three dimensional lattice of the cellular framework it has not been possible to extract them completely from this matrix without changing their chemical and molecular composition. For these reasons most of the conclusions based upon the role of pectic substances in fruit texture have been drawn from the characterization of less than 50 to about 70% of the total pectic substances present *(7)*.

The assumption that the unextracted pectic substances are identical to those extracted, isolated and analyzed is at best tenuous. Further work may or may not demonstrate that unextracted pectic substances have the same physical and chemical composition as those isolated.

The procedure outlined here permits a quantitative determination and partial chemical characterization of the total amount of pectic substances without extraction from the plant tissue. Fresh plant tissue was extracted with acidified ethanol which served to inactivate enzymes, to remove sugars, soluble organic acids and salts, and convert the pectinate salts to their acids. The resulting marc (insoluble residue) was analyzed directly without extracting the pectic substances. The carboxyl groups, free and combined, present in the insoluble sugar-free marc are those associated with the pectic substances. After corrections for acetyl, the polygalacturonic acid content analyzed by titration and by a colorimetric carbazole method were in agreement. All of the carboxyl groups present in the marc were titrated. The sum of the esterified uronic acid and acetyl carboxyl groups was obtained by direct saponification. The acetyl value was determined by a colorimetric procedure with the ferric hydroxamic acid color reaction with esters *(5)*. The total anhydrouronic acid, the acetyl, and percent uronic acids esterified were thereby obtained. Results can be obtained

* Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

on limited amounts of plant material in less working time, and without the uncertainty inherent in previously described methods.

EXPERIMENTAL

Preparation of marc. Add a representative sample of the fresh plant material to 3 volumes of 95% ethanol. This serves to inactivate the enzymes and partially dehydrate and extract soluble substances from the pulp. After at least one hour of the initial treatment, drain and filter off the solvent through a fine-porosity nylon cloth and press as dry as possible. Blend portions of the pulp in an electric blender with about two volumes of acidified ethanol (750 ml. of 95% ethanol, 200 ml. water, and 50 ml. of concentrated hydrochloric acid). Combine the blended mixture, stir vigorously for about an hour, and filter off the solvent as before. When the material becomes sufficiently dehydrated to filter easily, further filtrations and washings may be done on a coarse fritted glass funnel. Blend the pulp with two volumes of fresh 70% ethanol, filter and repeat this blending and washing treatment until portions of the filtrate show a negative reaction for chloride with a silver nitrate test.

Wash the moist chloride-free pulp once or twice with 2 volumes of acetone and dry the material without heating. Samples of the dried marc are much more easily handled if they are in moisture equilibrium with that prevailing in the laboratory. Grind the air-dried marc to pass a 40-mesh screen.

Free acidity *(9)*. Add to 1.000 g. of air-dried marc 5 ml. of ethanol, 100 ml. of water, and 1 g. of sodium chloride *(4)*. For ease of titration allow the slurry to shake for 15 minutes on a mechanical shaker. Add 6 drops of Hinton's indicator *(2)* (prepared by mixing the sodium salts of the following: 3 volumes of 0.4% phenol red and 1 volume each of 0.4% bromothymol blue and 0.4% cresol red, with 1 volume of distilled water) and titrate with 0.1*N* sodium hydroxide to a magenta end-point that holds for at least one minute. Shake the marc suspension vigorously during the titration. The titer in milliliters times the normality of sodium hydroxide equals milliequivalents of free carboxyl groups per gram of marc.

Total esters. Add 10 ml. of 0.5 *N* sodium hydroxide to this neutralized suspension and allow the mixture to stand for 30 minutes at room temperature to saponify the ester groups. Add exactly 10 ml. of 0.5 *N* hydrochloric acid and titrate the marc sample with 0.1 *N* sodium hydroxide to a Hinton's indicator magenta end-point that remains for one minute while shaking is continued. Correct this titer for inequalities in the 0.5 *N* reagents by running a blank. The corrected titer in milliliters times the normality of sodium hydroxide equals milliequivalents of esters saponified per gram of marc.

Acetyl. Determine acetyl directly on the dried fruit marc colorimetrically, with the hydroxamic acid method of McComb and McCready *(5)*.

Calculations. Reduce all measurements to milliequivalents per gram of marc. Then: Anhydrouronic acid (AUA) equivalent weight = 176.

Esters less acetyl = esterified uronic acid carboxyls.

Free acid plus esterified uronic acid carboxyls = total uronic acid carboxyls (free and combined)

$$\% \text{ anhydrouronic acid (AUA)} = \frac{176 \times 100 \times \text{total uronic acid carboxyls}}{1{,}000}$$

$$\% \text{ esterification} = \frac{\text{esterified uronic acid carboxyls} \times 100}{\text{total uronic acid carboxyls}}$$

RESULTS AND DISCUSSION

The results of the analyses of some fruit and vegetable marcs are presented in Table 1. The anhydrouronic acid (AUA) is compared with data obtained by a colorimetric carbazole method *(6)*.

The AUA determined colorimetrically is comparable to that calculated from the sum of the free and esterified uronic acid carboxyl groups in the proposed titrimetric method. These results indicate that in these samples of

TABLE 1
Analyses of fruit and sugar-beet marcs[1]

Sample	Ash	Water	Anhydrouronic acid		Acetyl	Degree of esterification
			Carbazole %	Titrimetric %	%	%
Blenheim apricots	0.41	10.21	24.9	26.6	1.46	69.4
Montmorency cherries	0.76	11.15	29.3	28.5	0.68	31.3
Royal Anne cherries	0.29	10.02	30.5	30.4	1.17	62.1
July Elberta peaches	1.39	9.94	22.3	23.7	1.37	73.6
Santa Rosa plums	0.42	10.62	23.3	21.6	1.31	72.2
Sugar beet	0.31	8.65	20.9	18.2	3.29	47.2
Sugar beet[2]	1.58	7.83	21.5	21.2	3.60	47.2

[1] The analyses are averages of 3 or more separate determinations that agreed well with each other.
[2] Sugar-beet marc prepared by washing with acetone in the same concentrations as that described using ethanol.

marc, with the exception of the acetyl esters, all of the carboxyl groups determined by titration, free and esterified, are those associated with the pectic substances. This procedure has been used before for analyzing partially purified pectic substances *(2)*. Anyas-Weisz *et al. (1)* used an ion exchange procedure to remove soluble ionic constituents prior to determining the pectic substances. When acetyl groups were present, they were determined separately and appropriate corrections made.

In the samples analyzed here the ash varied from 0.29% to 1.39% but the alkalinity of the ash was negligible and corrections were not required in calculating the anhydrouronic acid values as was previously recommended *(9)*.

Marc samples prepared as described using ethanol as a solvent to remove soluble materials should be analyzed as soon as practicable. Some samples stored at room temperature have shown a measurable change in conversion of esterified carboxyls to free carboxyls in one week's time. If it is desirable to store samples for a long period of time, further tests should be made, or preferably they should be stored in acetone in the semi-fresh condition and before acidification. Ethanol was used to prepare the fruit marc samples shown in Table 1, but acetone was substituted for ethanol in the preparation of a sample of sugar beet marc. The results shown in Table 1 indicate that the use of acetone is as effective as ethanol.

Furthermore, qualitative tests for pectin *in situ* with a modification of a hydroxamic acid test for pectin *(8)* have indicated that pectic substances in plant materials stored for long periods of time in ethanol undergo slow esterification. Esterification does not occur when acetone is used as a solvent to prepare marc.

The assumption that the titratable acidity present in all species of fruit and vegetable marc is due to pectic substances should not be made. The total pectic substances as determined by the carbazole method should agree with that calculated from the titrimetric procedure. If these results do not agree, the titrimetric procedure cannot be employed for study of the pectic substances in the material.

SUMMARY

A procedure is described whereby samples of fruit and vegetable marc are prepared by removing soluble substances with acidified dilute ethanol or

acetone. The marc is analyzed by titration for total carboxyl and after saponification for total ester groups. The acetyl is determined separately by a colorimetric method and the esterified uronic acid groups are calculated from total esters less acetyl.

Thus total pectic substances as anhydrouronic acid (AUA), the degree of esterification, and acetyl can all be obtained by direct analysis of marc without isolating and purifying the pectic materials, provided it has first been established that total pectic substances calculated by this method agree with results determined by some other method such as the carbazole method.

LITERATURE CITED

1. Anyas-Weisz, Solms, J., and Deuel, H. Bestimmung and Charakterisieurung von Pektinen mit Hilfe von Ionenaustauschern. *Mitt. Lebensmitt. Untersuch. Hyg.*, **42**, 91 (1951).
2. Hinton, C. L. *Fruit Pectins*. 1940. Chemical Publishing Co., New York, N. Y. pp. 27-34.
3. Kertesz, Z. I. *The Pectic Substances*. 1951. Interscience Publishers, Inc., New York, N. Y.
4. Linneweaver, H. Acceleration by electrolytes of alkaline de-esterification of pectin. *J. Am. Chem. Soc.*, **67**, 1292 (1945).
5. McComb, E. A. and McCready, R. M. Determination of acetyl in pectin and in acetylated carbohydrate polymers. *Anal. Chem.*, **29**, 819 (1957).
6. McCready, R. M., and McComb, E. A. Extraction and determination of total pectic materials in fruits. *Anal. Chem.*, **24**, 1986 (1952).
7. McCready, R. M., and McComb, E. A. Pectic constituents in ripe and unripe fruit. *Food Research*, **19**, 530 (1954).
8. McCready, R. M., and Reeve, R. M. Test for pectin based on reaction of hydroxamic acids with ferric ion. *J. Agr. Food Chem.*, **3**, 260 (1955).
9. Owens, H. S., McCready, R. M., Shepherd, A. D., Schultz, T. H., Pippen, E. L., Swenson, H. A., Miers, J. C., Erlandsen, R. F., and Maclay, W. D. Methods used at Western Regional Research Laboratory for extraction and analysis of pectic materials. U. S. Dept. Agriculture, Bureau Agr. Ind. Chem., *AIC-340* (1952).

EFFECT OF CERTAIN ION COMBINATIONS COMMONLY FOUND IN POTABLE WATER ON RATE OF FILTRATION THROUGH ROASTED AND GROUND COFFEE

D. G. GARDNER

United States Testing Company, Inc., Hoboken, New Jersey

(Manuscript received June 2, 1957)

In spite of the widespread popularity of coffee as a beverage, there is little agreement about the effect of water on its quality. In addition, there is no evidence to support any of the opinions advanced. The same coffee brewed in neighboring localities, where the composition of the water is the only variable, will yield beverages of highly different quality. It is logical to assume that variations in the coffee prepared in different parts of the country might be due to the relatively large differences in the composition of the water used. Table 1 *(4)* shows the composition of the water supplies of several different cities in the United States.

TABLE 1 (4)
Composition of the Water Supplies of Different Cities in the United States[1]

	Boston Mass.	New York N. Y.	Chicago Ill.	Los Angeles Calif.	New Orleans La.	Houston Texas
Iron	0.12	0.20	0.20	0.04	.....	0.17
Calcium	4.4	13.0	34.0	27.0	17.0	22.0
Magnesium	1.0	4.5	9.7	12.0	7.1	5.8
Sodium	3.9	3.3	5.1	74.0	23.0	108.0
Potassium		1.3				1.9
Bicarbonate	16.0	42.0	146.0	215.0	45.0	301.0
Sulfate	9.8	17.0	12.0	39.0	43.0	7.8
Chloride	3.2	4.0	4.5	44.0	28.0	48.0

[1] All data in p.p.m.

Water is important to flavor, but it is not the *direct* cause of flavor variations. In other words, the ions normally found in drinking water must be present in exceptionally high concentrations to contribute directly to the flavor of the beverage *(6)*. Therefore, the cause of perceptible differences in coffee brewed with water from different localities must be a function of some *indirect* effect of the ions present. One such effect might be the time of exposure of coffee to water.

Prescott reports that extended brewing time produces deleterious effects on the final beverage *(8)*. He attributes the resulting unacceptability to an over-extraction characterized by excessive bitterness and astringency. Extraction, in turn, is affected by time of contact of water with grounds. Combining these facts, we concluded that we should first concern ourselves with the time of brewing as a major factor.

Only one brief report on the effects of water composition and water treatment on brewing time and acceptability has appeared in the literature *(1)*. A summary of the study on which this report was based, and from which no experimental data were ever published, states that coffee made with distilled

water was considered less desirable than that made with very hard, untreated water. Hard water, softened, made poorer coffee than untreated water. Treated water caused an increase in preparation time which in turn decreased acceptability.

Although water composition undoubtedly influences beverage composition, flavor, and appearance, only its effect on brewing time was studied at this time. Subsequent investigations will provide knowledge relating all of the factors affecting the quality and acceptability of beverage coffee.

EXPERIMENTAL

In order to eliminate all variables not directly under study, the filtration procedure was standardized as follows:

1. The brewing technique selected was the drip method—in essence, a filtration of heated water through a bed of roasted and ground coffee. The same piece of equipment was used in all tests (Figure 5).
2. A quantity of roasted and ground coffee sufficient for all tests was composited and tumble-mixed to insure homogeneity of the individual samples.
3. All water employed in the preparation of specific ion solutions was first deionized by passage through an ion exchange resin. Data for the purity of the water is given in Table 2.

TABLE 2
Analysis of Deionized Water

Chemical Analysis of Water		Spectrographic Analysis of Residue[1]	
Determination	Concentration	Determination	Concentration
Residue, mineral	1 p.p.m.	Sodium	trace
Hardness (as $CaCO_3$)	nil	Boron	faint trace
pH	5.3	Barium	not found
Carbonate (as $CO_3^{=}$)	nil	Bismuth	not found
Bicarbonate (as HCO_3^{-})	6.1 p.p.m.	Chromium	not found
Iron	0.1 p.p.m.	Manganese	faint trace
Aluminum	nil	Molybdenum	not found
Silica (SiO_2)	nil	Nickel	faint trace
Calcium	nil	Lead	faint trace
Magnesium	nil	Tin	faint trace
Chloride	0.3 p.p.m.	Titanium	trace
Sulfate	0.4 p.p.m.		

[1] Spectrographic analysis is reported on the mineral residue of 1 p.p.m. as follows:
Trace — 0.01 - 0.1%.
Faint Trace — less than 0.01%

Procedure for measuring total brewing time and brewing rate. Seventy-three grams of coffee were placed in the lower drip section of an 8-cup drip coffee maker. The coffee maker was suspended over a 1000 ml. graduate. A glass funnel was attached to the lower drip section of the coffee maker so that the filtrate could be collected and measured in the graduate (Figure 5).

Eleven hundred milliliters of water were brought to boiling and then quickly poured onto the bed of coffee in the coffee maker. The time in seconds required to collect each 50 ml. of filtrate was recorded up to and including 900 ml. Timing commenced upon the addition of the water. The time required to collect each 50 ml. of filtrate determines the brewing rate. The time required to collect 900 ml. of filtrate is referred to as the total brewing time.

RESULTS AND DISCUSSION

The standard brewing time against which all variations could be compared was established with deionized water. Eight determinations of the total brewing time gave an average of 350 seconds; the standard deviation of measurement was 9 seconds, and the range was 337 to 366.

After a standard total brewing time had been established, solutions containing known amounts of 16 different inorganic compounds were added and the effect on total brewing time observed. In the first set of experiments, the solutions each contained 400 p.p.m. of a single inorganic compound in deionized water. Triplicate determinations were made of the total brewing times of these 16 solutions. The average total brewing times for each of the 16 solutions are given in Figure 1. It will be noted that the effect of the different ions on the

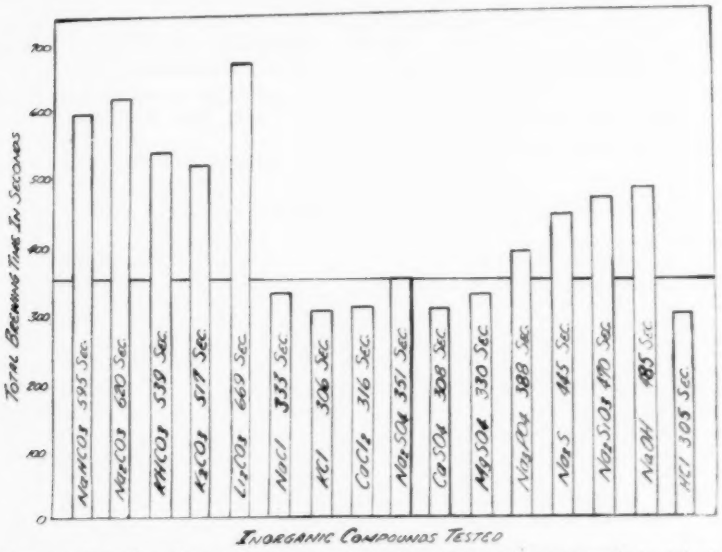


Figure 1. Brewing times of 400 p.p.m. of various inorganic compounds in deionized water. Reference line at 350 seconds is brewing time using pure deionized water.

total brewing time is variable. In the case of lithium carbonate the time is nearly doubled. In some instances, the addition of ions decreased the brewing time slightly below the standard. Carbonates and bicarbonates as a group appear to have the greatest retarding effect, while all other types normally present in municipal water supplies appear to have little effect. Additional experiments (Figure 2) showed that the brewing time was greatly changed by increase in the concentration of bicarbonate, but not by increasing the concentration of other ions. Figure 3 illustrates the change in brewing rate when the concentration of the bicarbonate is varied. It should be noted that the greatest change in rate occurs during the first portion of the brewing cycle.

The data obtained up to this point in the investigation suggest an interesting

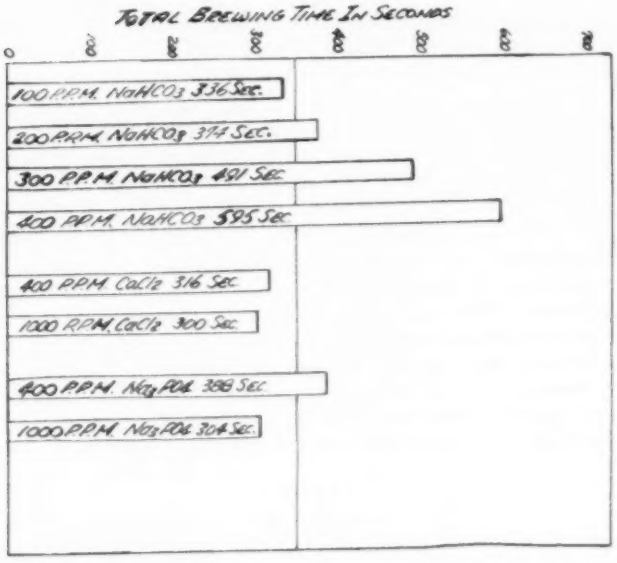

TOTAL BREWING TIME IN SECONDS
100 P.P.M. NaHCO3 336 SEC.
200 P.P.M. NaHCO3 374 SEC.
300 P.P.M. NaHCO3 491 SEC.
400 P.P.M. NaHCO3 595 SEC.
400 P.P.M. CaCl2 316 SEC.
1000 P.P.M. CaCl2 300 SEC.
400 P.P.M. Na2PO4 398 SEC.
1000 P.P.M. Na3PO4 304 SEC.

Figure 2. Effect on total brewing time of varying concentrations of compounds in solution. Line at 350 seconds is brewing time using pure deionized water.

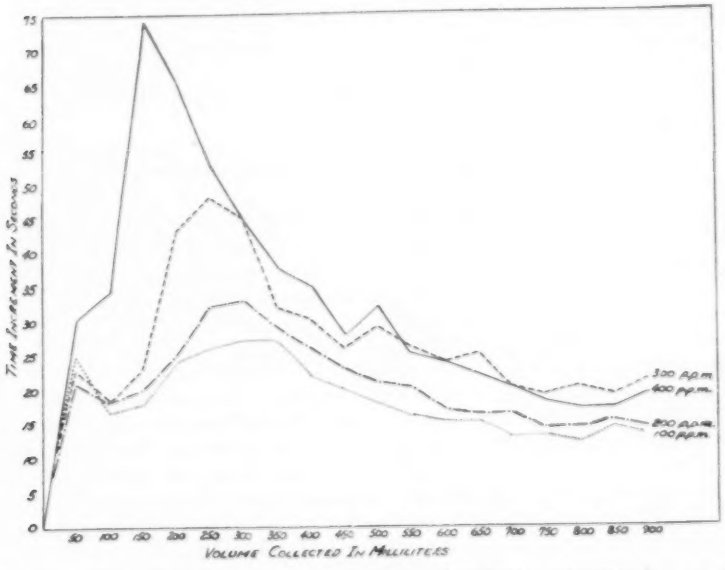


Figure 3. The effect of varying the sodium bicarbonate concentration on the brewing rate.

hypothesis: One commonly used water softening process replaces the calcium ions with sodium *(3)*. In this form of softening there will be little or no change in the carbonate or bicarbonate content, but a very large increase in the concentration of sodium.

In view of the evidence presented above, it is possible that a carbonate hard water softened by sodium exchange process would significantly increase the brewing time. In order to verify this theory, hard water was prepared by adding a calcium carbonate solution to deionized water. The calcium carbonate solution was prepared by dissolving calcium carbonate in dilute hydrochloric acid and then neutralized with ammonium hydroxide.

The solution was then saturated with carbon dioxide to increase bicarbonate ion concentration. This water, containing only carbonate or temporary hardness, was softened by passage through a zeolite-type water softener. The average total brewing times, as well as analytical data on the hard and softened water are listed in Table 3. Softening increased brewing time from 349 to 466 seconds. An increase in the concentration of carbonate or bicarbonate ions does cause an increase in the filtration time of water through coffee.

TABLE 3
Experiment 1

	Hard Water	Softened Water
pH	6.6	7.6
Hardness (as $CaCO_3$) (*)	524 p.p.m.	30 p.p.m.
Sodium (as NaCl) (**)	8 p.p.m.	847 p.p.m.
Potassium (as KCl) (**)	nil	0.5 p.p.m.
Carbonate (as $CO_3^=$) (***)	nil	nil
Bicarbonate (as HCO_3^-) (***)	162 p.p.m.	272 p.p.m.
Total Brewing Time Using Hard Water	.....	349 seconds
Total Brewing Time Using Softened Water	.....	466 seconds

Legend:
* by Betz titration *(2)*.
** by flame photometry.
*** by titration with sulfuric acid to phenolphthalein and methyl orange end-points.

Another type of hard water contains calcium, magnesium and sulfate ions. To determine whether or not non-carbonate, or permanent hardness, of this type after softening has any effect on brewing time, two solutions containing 400 p.p.m. of calcium sulfate and magnesium sulfate were prepared. Half of each solution was run through the softener and the total brewing time for each was measured. The data for these two experiments are given in Table 4. Very little difference was found between the total brewing times of the calcium and magnesium solutions before and after softening. This is to be expected. When solutions of magnesium and calcium sulfate are put through a zeolite exchanger, the calcium and magnesium ions are exchanged for sodium ions. The effluent from the softener in each case will be a solution of sodium sulfate. The initial tests made in this study have shown that a 400 p.p.m. solution of sodium sulfate will have a total brewing time equal to that of deionized water and about equal to that of 400 p.p.m. solutions of calcium sulfate and magnesium sulfate.

To show that the results obtained from experiments with synthetic waters apply to practical situations, samples of drinking water were collected from 8

TABLE 4(a)
Experiment 2-400 P.P.M. $CaSO_4$

	Hard Water	Softened Water
pH	6.9	7.0
Hardness (as $CaCO_3$) (*)	294.0 p.p.m.	7.6 p.p.m.
Sodium (as NaCl) (**)	3.5 p.p.m.	380.0 p.p.m.
Potassium (as KCl) (**)	nil	nil
Carbonate (as $CO_3^=$) (***)	nil	nil
Bicarbonate (as HCO_3^-) (***)	17.1 p.p.m.	36.6 p.p.m.
Alkalinity (as OH^-) (***)	nil	nil
Total Brewing Time Using Hard Water	.....	304 seconds
Total Brewing Time Using Softened Water	.....	332 seconds

Legend:
* by Betz titration *(2)*.
** by flame photometry.
*** by titration with sulfuric acid to phenolphthalein and methyl orange end-points.

TABLE 4(b)
Experiment 3-400 P.P.M. $MgSO_4$

	Hard Water	Softened Water
pH	5.4	7.3
Hardness (as $CaCO_3$) (*)	390.0 p.p.m.	13.0 p.p.m
Sodium (as NaCl) (**)	nil	485.0 p.p.m.
Potassium (as KCl) (**)	nil	nil
Carbonate (as $CO_3^=$) (***)	nil	nil
Bicarbonate (as HCO_3^-) (***)	12.2 p.p.m.	84.8 p.p.m.
Alkalinity (as OH^-) (***)	nil	nil
Total Brewing Time Using Hard Water	.....	261 seconds
Total Brewing Time Using Softened Water	.....	323 seconds

Legend:
* by Betz titration *(2)*.
** by flame photometry.
*** by titration with sulfuric acid to phenolphthalein and methyl orange end-points.

cities distributed geographically throughout the United States. These waters were analyzed and the findings are reported in Table 5. Four of these waters are taken from areas in which softening is the usual practice. A portion of these waters were softened with the zeolite equipment and reanalyzed (Table 5). The effect of treatment on the 4 waters is presented graphically in Figure 4. In every case, treatment resulted in an increase in brewing time in proportion to the amount of carbonate hardness present in the original water.

SUMMARY AND CONCLUSIONS

Coffee beverage of high quality, like every other food product, requires careful preparation from high quality ingredients. Recognized as an important ingredient of variable composition, water does change beverage quality adversely—so frequently that its function and influence must be studied carefully from a number of aspects. One of these, the effect of water composition on preparation time in a percolation system, was the subject of this report.

The investigation showed that most of the ions present in many water supplies change the brewing time only slightly. However, the presence of

TABLE 5
City Waters as Received

	pH	Hardness (as $CaCO_3$) p.p.m.	Sodium (as NaCl) p.p.m.	Potassium (as KCl) p.p.m.	Carbonate (as $CO_3=$) p.p.m.	Bicarbonate (as HCO_3-) p.p.m.
Dallas, Texas	8.6	421	299	3	nil	439
Los Angeles, Calif.	9.1	266	115	7	nil	210
Denver, Colo.	8.5	116	64	3	nil	89
Memphis, Tenn.	8.9	63	29	2	nil	94
Philadelphia, Pa.	8.1	89	27	4	nil	45
Woonsocket, R. I.	5.0	16	7	1	nil	11
Providence, R. I.	8.3	33	9	1	6	18
Boston, Mass.	7.0	16	7	1	nil	14
Four City Waters After Softening						
Dallas, Texas	9.2	18	963	2	nil	461
Los Angeles, Calif.	9.5	9	700	5	nil	306
Denver, Colo.	9.6	6	350	3	nil	77
Philadelphia, Pa.	7.8	7	155	1	nil	75

carbonate or bicarbonate ions increase this time significantly and in direct relation to their concentration. This effect is accentuated by sodium ions if water, containing temporary hardness and softened in a zeolite conditioner, is used. Water, classified as permanently hard, whether treated or not, had little effect on preparation time.

Time of contact between coffee grounds and water is one of the most

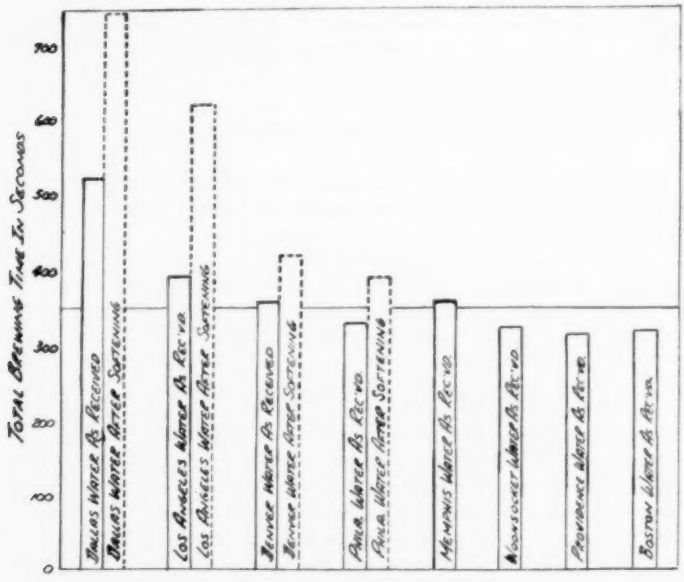


Figure 4. Effect of Zeolite softening process on the total brewing time of carbonate hard waters. Line at 350 seconds is brewing time using pure deonized water.

important factors affecting the quality of beverage. An extraction of the grounds ranging from 18 to 22% is most desirable *(7)*. Too long a time of contact in relation to the equipment and grind used causes over-extraction ranging from 22 to 30%, and bitterness *(5)*. Consequently, water containing a high concentration of bicarbonate and excessive sodium resulting from zeolite treatment, will favor over-extraction and unacceptability. To avoid this possi-

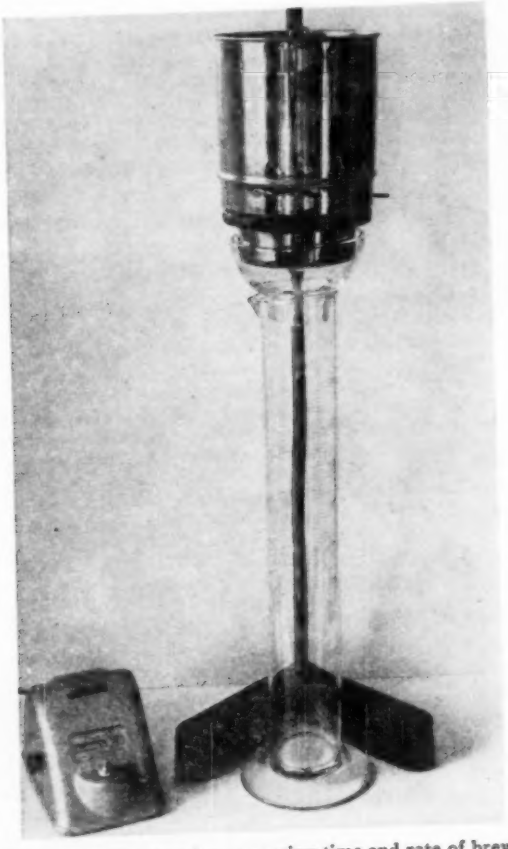


Figure 5. Apparatus for measuring time and rate of brewing.

bility other types of water treatment than those using only sodium exchange materials should be developed and adapted to coffee making if water analysis indicates conditioning is necessary.

Acknowledgment

This work was supported by a grant from the Coffee Brewing Institute, Inc.

LITERATURE CITED

1. Aborn, E., *Tea and Coffee Trade J.*, **95**, (4) 28, (1948).
2. Betz, W. H. and Betz, L. D., *Betz Handbook of Industrial Water Conditioning*, 3rd ed., 1950. W. H. & L. D. Betz, Philadelphia, Pa. pp. 153-155
3. Fair, G. M. and Geyer, J. C., *Water Supply and Waste-Water Disposal*, 1954. J. Wiley, New York, N. Y. pp. 625-31.
4. Lange, N. A., *Handbook of Chemistry*, 8th ed., 1952. Handbook Publishers, Inc., Sandusky, Ohio. pp. 786-788.
5. Lockhart, E. E., The soluble solids in beverage coffee as an index to cup quality. *Tea and Coffee Trade J.*, **113**, (1) 12, (1957); *Coffee and Tea Industries*, **80**, (7) 16, (1957).
6. Lockhart, E. E., Tucker, C. L. and Merritt, M. C., The effect of water impurities on the flavor of brewed coffee, *Food Research*, **20**, 598-605 (1955).
7. Niven, W. W. Jr., and Shaw, B. C., *Tea and Coffee Trade J.*, **112**, (4) 28, (1957); Coffee and Tea Industries, **80**, (4) 44, (1957).
8. Prescott, S. C., Joint Coffee Trade Publicity Committee. 1920-23. New York, N. Y.

THE EFFECT OF BLANCHING ON THE CARBONYL CONTENT OF THE CRUDE LIPID DURING THE STORAGE OF FROZEN PEAS[a]

FRANK A. LEE

New York State Agricultural Experiment Station, Cornell University, Geneva, N. Y.

(Manuscript received June 8, 1957)

In previous papers from the author's laboratory *(3, 4, 5)* chemical changes taking place in the crude lipid during the storage of vegetables have been reported. These studies showed that peroxides and acids develop in the lipids of peas and some other vegetables under these conditions. The present study is concerned with the content of carbonyl compounds in the crude lipid of peas, prepared from blanched and unblanched peas which had been held in frozen storage, and from the fresh vegetable.

MATERIALS AND METHODS

Peas of the Thomas Laxton variety, both raw and blanched from the same lot, which had been held in storage at —17.8°C. for 5½ years, were used. In addition to these samples, fresh peas were purchased on the market for a study of the carbonyl content of the crude lipids extracted from raw and blanched fresh peas. The variety of these peas was unknown, but they appeared to be of the Telephone type. It must be stated, however, that these peas came from a distance, and, therefore, the time which elapsed between harvest and the laboratory work could have been comparatively long. These samples were dried by lyophilization and the lipid material extracted with anhydrous peroxide-free diethyl ether, as described in previous publications.

The samples were analyzed for unsaturated and saturated carbonyl compounds by the method of Henick, Benca, and Mitchell *(2)* as modified by Chipault and associates *(1)* of the Hormel Institute, Austin, Minnesota.

RESULTS AND DISCUSSION

It can be seen (Table 1) that unsaturated carbonyl content is not evident in the fresh material, nor in the older material blanched previous to storage. It is quite evident, therefore, that there is a connection between the development in the peroxides as noted in previous publications *(3, 4, 5)* and the formation of unsaturated carbonyl compounds. It must be remembered, however, that the method of analysis employed measures as unsaturated carbonyl content only carbonyl compounds with the double bonds conjugated with the carbonyl group. A further test was made by blanching a sample of the raw stored material by plunging the frozen material into boiling water, and boiling for two minutes previous to freezing for lyophilization. After lyophilization, the lipid was extracted and used for carbonyl analyses. The unsaturated carbonyl was slightly lower than that present in the corresponding raw material.

Actually, the results given as zero for unsaturated carbonyl compounds were small negative values, except in the lipids extracted from raw and blanched fresh peas, in which cases larger negative values were obtained, but

[a] Approved by the director of the New York State Agricultural Experiment Station for publication as Journal Paper No. *1081*.

TABLE 1
Carbonyl Content of Crude Pea Liquid

Sample	Carbonyl Content[1]	
	Unsaturated	Saturated
Lipid from raw peas[2]	67.8	78.5
Lipid from blanched peas[2]	0	96.6
Lipid from fresh market peas	0	109.2
Lipid from fresh market peas, blanched prior to lyophilization and extraction of crude lipid	0	163.2

[1] All determinations run in triplicate. Expressed as millimoles of carbonyl compounds per kilogram of sample.
[2] Peas were held for 5½ years at −17.8°C. before extraction of crude lipid.

it seemed reasonable to report them as zero. It is likely that the method is less accurate at the lower end of the scale.

It seems quite apparent that unsaturated carbonyl compounds are formed in the raw material during storage at —17.8° C. for prolonged periods of time. The progressive development of these compounds during storage is not known at present.

Saturated carbonyl compounds were higher in the crude lipid extracted from the blanched peas than in that extracted from the corresponding raw peas, fresh or stored.

SUMMARY

Crude lipids, prepared from frozen peas stored for 5½ years, both raw and blanched, and from fresh peas, both raw and blanched were chemically examined for saturated and unsaturated carbonyl compounds.

Peas held in storage unblanched yielded crude lipids which contained appreciable amounts of unsaturated carbonyl compounds. Unsaturated carbonyl compounds were not found in the crude lipids extracted from material blanched previous to storage.

Crude lipids extracted from fresh market peas, raw or blanched, contained no unsaturated carbonyl compounds.

LITERATURE CITED

1. CHIPAULT, J. R. (Personal communication).
2. HENICK, A. S., BENCA, M. F., AND MITCHELL, J. H. *J. Am. Oil Chemists' Soc.*, **31**, 88-91 (1954). *Ibid.* **33**, 35 (1956) (for correction).
3. LEE, FRANK A. Chemical changes taking place in the crude lipids during the storage of frozen raw vegetables. *Food Research*, **19**, 515-20 (1954).
4. LEE, FRANK A., AND WAGENKNECHT, A. C. On the development of off-flavor during the storage of frozen raw peas. *Food Research*, **16**, 239-44 (1951).
5. LEE, FRANK A., WAGENKNECHT, A. C. AND HENING, J. C. A chemical study of the progressive development of off-flavor in frozen raw vegetables. *Food Research*, **20**, 289-97 (1955).

ROASTING OF FROZEN STUFFED TURKEYS. I. SURVIVAL OF *SALMONELLA PULLORUM* IN INOCULATED STUFFING

RAYMOND E. ROGERS AND MILLARD F. GUNDERSON

Research Department, Campbell Soup Company, Omaha, Nebraska

(Manuscript received July 11, 1957)

In our present-day society there has arisen a situation where not all of us are directly involved in the production of the articles we consume. This is becoming more and more true in the production and control of food supply. New procedures in processing, storage, transportation, and preparing foods have been brought about. Despite the elaborate precautions taken, the possibility of bacteriological development may be enhanced by mishandling of an otherwise excellent product.

In order to prevent food-borne infections, it is necessary to prevent the entrance or development of these types of bacteria in foods. Some foods may be contaminated during processing and preparation; other foods, such as meat products, may harbor these organisms from the animal. Food handlers and cooks carry potentially dangerous bacteria on their hands as well as in their respiratory tracts. Rodents and also insects such as flies and cockroaches are carriers of bacteria. It is practically impossible to be certain that these bacteria are eliminated from food; therefore, foods should be treated in some way to destroy them, and then held under conditions which will prevent their recontamination and growth.

In the studies on the possibility of the transmission of *Salmonella* infections by eating cooked food containing viable *Salmonella* organisms, Husseman and Wallace *(3)* found that artificially infected chickens cooked in the laboratory were not rendered free from these bacteria. The degree of cooking for the chickens was judged by timing. The total time of cooking for the broilers was 41 minutes; for the roasters 35 minutes per pound was used. The oven regulator was set at 325° F. Thermal resistance studies by Webster and Esselen *(8)* of *Salmonella enteritidis* in poultry stuffing showed that the organisms survived 5 minutes at 145° F. and 6 minutes at 140° F. when the initial concentration of organisms in the stuffing was 50,000 to 100,000 organisms per ml. Castellani *et al. (1)* in a study of the roasting of stuffed turkeys found that present roasting processes appear to be generally adequate for smaller birds; but for birds over 18 pounds, there was little if any margin of safety in some recommendations. After their study, Esselen *et al. (2)* stated that the roasting time required for good quality as judged by appearance, texture, and flavor exceeds the minimum time required to destroy food poisoning organisms.

In view of the role played by poultry in *Salmonella* food-borne infections, it was the purpose of this investigation to learn something about the time and temperature required to render a stuffing free from *Salmonella pullorum*.

EXPERIMENTAL PROCEDURE

Culture. The *Salmonella pullorum* culture employed in this investigation was obtained from the University of Nebraska Medical School culture collection. No information was available on this culture. The culture was carried on brain heart infusion agar slants and transferred bi-weekly. Forty-eight hours previous to the inoculation of the stuffing, the culture was transferred and incubated for 24 hours at 37° C. Twenty-four hours previous to the inoculation of the stuffing the culture was transferred from the slants to brain heart infusion broth and incubated at 37° C. This broth culture was used to inoculate the stuffing. A new batch of inoculum was prepared for the inoculation of the stuffing for both the medium and the large size birds.

Inoculation and stuffing. Nine frozen unstuffed turkeys with weights ranging from 9.7 to 10.13 pounds, and 7 with weights ranging from 17.4 to 18.10 pounds were placed in watertight plastic bags, sealed, and submerged in water to thaw. The thawed turkeys were removed from the water, the plastic bags discarded, and the giblets removed from the body cavity. Each turkey was weighed. The turkeys were stuffed and the body cavities closed by means of metal skewers. The turkeys were again weighed and the amount of stuffing each bird contained calculated by difference. The turkeys were inserted into plastic bags, sealed, and immediately placed in a –40° F. to –50° F. wind tunnel. After completely frozen, the turkeys were stored in a storage room with a temperature of –9.4° F. The turkeys remained in storage from 2 to 10 days before testing. A determination of the number of *Salmonella pullorum* organisms per gram of stuffing was not made immediately prior to roasting. The number of organisms was presumed to be the same as at the time of inoculation.

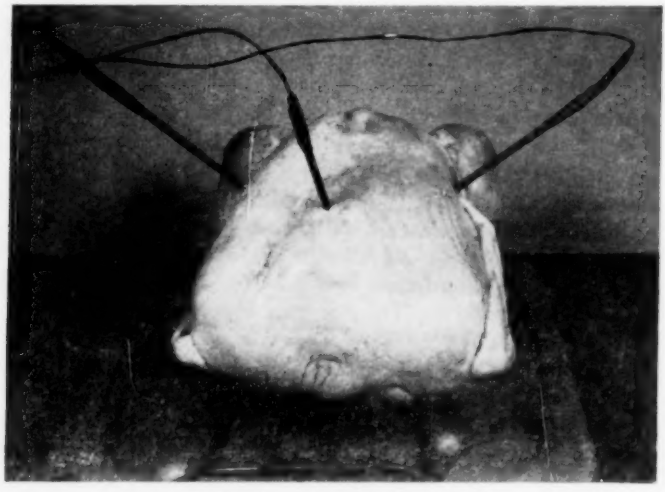


Figure 1. Frozen stuffed turkey showing the method of insertion of ceramic thermocouples.

A total bacterial count was made on duplicate 11 g. samples of frozen, uninoculated commercial stuffing. The stuffing was placed in a large pan, broken up, and mixed by hand with a 24-hour brain heart infusion culture of *Salmonella pullorum*. The inoculum was dispensed at the approximate concentration of 15 ml. of culture for each 50 g. of stuffing inoculated. Duplicate samples of the inoculated stuffing were tested to determine the number of *Salmonella pullorum* organisms per gram of stuffing.

Roasting procedure. Work done previously by Castellani *et al. (1)* indicated that

the spot which heats least rapidly is the approximate geometric center of the stuffed bird. Stuffing, however, differs in composition, consistency, and amount per bird. Preliminary studies were therefore done by Rogers and Johnson *(5)* on the heating characteristics of frozen stuffed turkeys to determine this "coldspot."

As each turkey was prepared for testing the plastic bag was discarded and the turkey was placed on a metal roasting pan. An electric drill was employed to drill holes into the turkey at an angle so that when the copper constantan thermocouples were inserted, the tips would be in the approximate geometric center of the stuffing. After waiting for a period of 5 minutes to allow the thermocouples equilibrate with the stuffing temperature, a reading was made previous to placing the turkey in the oven.

Two thermocouples had been previously placed in the oven. Both were in the approximate center of the oven. One was close to the top of the turkey, and one was close to the bottom of the turkey (Fig. 1).

To determine the temperature of the oven and the center of the stuffing before and throughout the roasting process a Leeds and Northrup potentiometer equipped with a calibrated temperature scale was employed (Fig. 2). A reading was made every 10 minutes during the roasting of the medium size turkeys and every 15 minutes during the roasting of the large size turkeys. Each turkey was removed from the oven at a designated temperature for testing and analyzing.

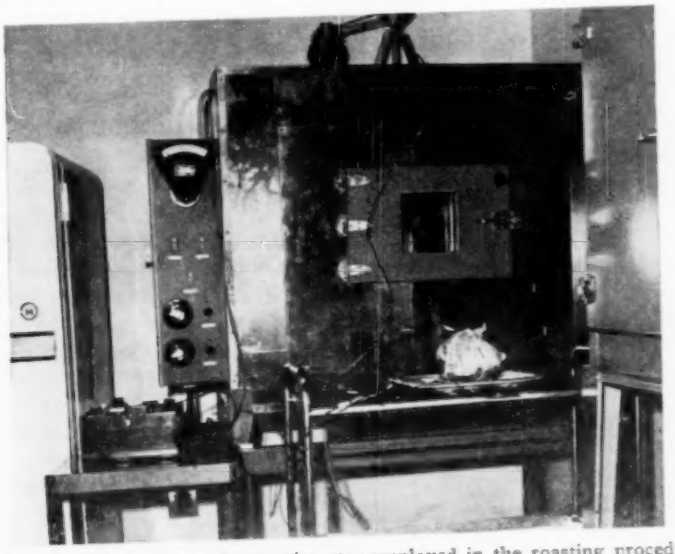


Figure 2. Test oven and potentiometer employed in the roasting procedure.

Bacteriological methods. Duplicate 11 g. samples of frozen uninoculated stuffing were macerated by a Waring blendor in 99 ml. of sterile distilled water. Standard plate counts were made by plating on tryptone glucose extract agar. Plates were incubated at 37° C. for 24 hours.

The number of *Salmonella pullorum* organisms per gram of the inoculated, unroasted and roasted stuffing was determined by the Most Probable Number technique. Five tubes of Salenite F broth were used for each dilution. After 48 hours incubation at 37° C. the tubes were streaked on brilliant green agar plates to determine growth. The plates were incubated for 24 hours at 37° C. The number of *Salmonella pullorum* organisms per gm. of sample was determined by the tables given in "Standard Methods for the Examination of Water, Sewage, and Industrial Waste." *(6)*

TABLE 1

Survival of *Salmonella pullorum* in stuffing of roasted, medium size frozen stuffed turkeys[1]

Turkey No.	Weight (lbs) of Stuffed Bird	Weight (lbs) of Stuffing	Initial Temp. (°F.) of Stuffing	Roasting Time at 325° F.	Highest °F. Temp. of Cent. of Stuffing	Survival of *Salmonella*/gm of stuffing as determined by MPN
5	10.90	2.60	29	2 hrs. 25 min.	75	31,000,000
8	10.80	2.90	0	3 hrs. 40 min.	100	14,000,000
3	10.50	2.30	0	4 hrs. 15 min.	125	4,900,000
2	10.70	2.50	0	4 hrs. 20 min.	135	1,100,000
4	10.90	2.90	0	4 hrs. 35 min.	145	26,000
9	9.70	2.30	4	4 hrs.	150	17,000
1	10.00	2.60	4	4 hrs. 20 min.	155	33
7	10.13	3.00	0	4 hrs. 55 min.	160	Less than 1.8 org/gm of sample
6	10.10	2.10	28	4 hrs. 35 min.	165	Less than 1.8 org/gm of sample

[1] Initial count of inoculated stuffing was 115,000,000 organisms per gram of stuffing.

RESULTS AND DISCUSSION

The relationship of time and temperature required to kill *Salmonella pullorum* during the roasting of medium-size frozen stuffed turkeys is shown in Table 1. It can be seen that with the stuffing possessing an initial count of 115,000,000 organisms per gram, a minimum temperature of 160° F. must be reached in the center of the stuffing to assure adequate destruction of the organisms. It required a period of 4 hours and 55 minutes to reach this lethal stuffing temperature when the initial temperature of the stuffing was 0° F.

The relation of time and temperature to the destruction of *Salmonella pullorum* during the roasting of large frozen stuffed turkeys is shown in Table 2. A minimum temperature of 155° F. as shown must be reached in the center of the stuffing with the initial count of the stuffing being 323,000,000 organisms per gram. A roasting period of 6 hours and 30 minutes was required for the complete destruction of the bacteria.

Castellani *et al. (1)* noted that a temperature of 165° F. reached in the center of the stuffing during the roasting period appeared sufficient to kill *Salmonella* organisms and allow a modest margin of safety. In no instance in their tests did the initial count of the inoculated stuffing exceed 5,000,000 organisms per gram, and a temperature at the center of the stuffing of 152° F. was sufficient to destroy the *Salmonella* organisms present in the stuffing. However, when a sample was removed from the center of the stuffing after a temperature of 140° F. had been attained, it was found that more than 50,000 *Salmonella* organisms survived per gram. Esselen *et al. (2)* found that larger birds (12 to 15 pounds) have a slower rate of heat penetration and require a longer cooking time. They recommend that a center temperature of 155° F. be attained before removing the bird from the oven. For birds less than 12 pounds which heat more rapidly, a center temperature of 165° F. should be reached. Their recommendations for cooking frozen birds required no special precautions other than to roast to what is considered to be a palatable and attractive degree of doneness and a center temperature as indicated for the different weight classes. The work of Tischer and Hurwicz *(7)* indicates that a more drastic heat treatment is required to destroy a large number of cells than a few. The results of Table 1 show that when the initial stuffing count was 115,000,000 organisms per gram, a temperature of 160° F. was required to destroy all the *Salmonella pullorum*. These observations are important in food preservation, for this is the basis for efforts to keep the number of bacteria in foods low.

It may also be seen from the results of Tables 1 and 2 that the initial temperature of the stuffing and the size bird (shown graphically in Figure 3) influenced the time required to reach the test temperatures. In Table 1 it required only 4 hours to reach a temperature of 150° F. in turkey Number 9, when the initial temperature of the stuffing was 4° F., and it required 4 hours and 15 minutes to reach a temperature of only 125° F. when the initial temperature was 0° F. The weight of both turkeys was approximately the same. In the roasting of turkey Number 5, it required only 4 hours and 28 minutes to reach a temperature of 155° F. when the initial temperature was 4° F. In Table 2 it required 6 hours and 15 minutes to reach 160° F. when the initial temperature

TABLE 2

Survival of *Salmonella pullorum* in stuffing of roasted, large size frozen stuffed turkeys[1]

Turkey No.	Weight (lbs) of Stuffed Bird	Weight (lbs) of Stuffing	Initial Temp. (°F.) of Stuffing	Roasting Time at 325° F.	Highest °F. Temp. of Cent. of Stuffing	Survival of *Salmonella*/gm of stuffing as determined by MPN
8	18.60	4.00	−3	5 hrs. 10 min.	100	17,000,000
7	17.40	3.80	−1	5 hrs. 20 min.	135	46,000,000
6	18.10	3.15	−2	5 hrs. 30 min.	140	6,800,000
2	18.50	3.14	−1	6 hrs.	145	4,900,000
4	18.00	3.12	0	6 hrs. 20 min.	150	240,000
1	18.00	3.14	0	6 hrs. 30 min.	155	Less than 1.8 org/gm of sample
5	18.40	3.10	3	6 hrs. 15 min.	160	Less than 1.8 org/gm of sample

[1] Initial count of inoculated stuffing was 323,000,000 organisms per gram of stuffing.

of the stuffing was 30° F.; yet, it took 6 hours and 20 minutes to reach a temperature of 150° F. when the initial stuffing temperature was 0° F.

It would seem evident that the roasting process of a turkey should not be interrupted. As shown in the results of Table 1, *Salmonella pullorum* organisms survived a temperature of 155° F. when the stuffing has a bacterial count of 33 organisms per gram. The practice of partially roasting a turkey would place the bird in what Jensen *(4)* called the "incubation danger zone." He found that food remaining between the temperatures of 50° F. and 118.4° F. for a period of from 4 to 8 hours was potentially dangerous. To hold a partially roasted turkey to complete the roasting later would place the bird in this zone at least

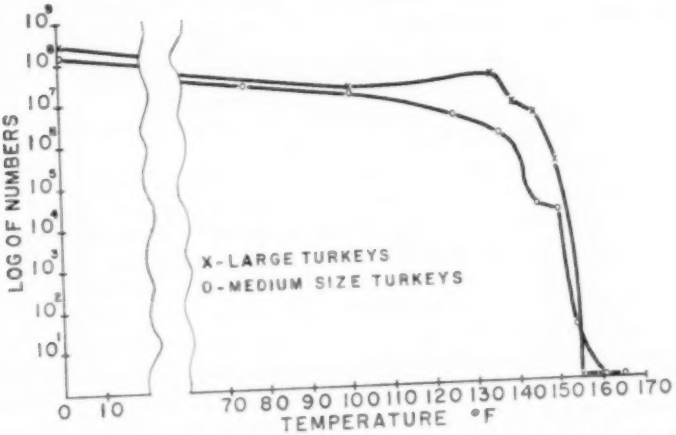


Figure 3. Survival of *Salmonella pullorum* in the stuffing during roasting of medium and large frozen turkeys.

three times: (a) during the initial roasting; (b) during the initial cooling, and (c) during the second roasting.

From an initial stuffing count of 323,000,000 organisms per gram in the large turkeys, at 100° F. the bacterial count had decreased to 17,000,000 organisms per gram. This temperature range is not believed to be lethal, as might be indicated. A bacterial count was not made on the stuffing immediately prior to the roasting of the turkeys. This decrease in count from the initial stuffing count might possibly be due to the fact that a decrease in the number of organisms occurred in process of freezing and storage after the initial inoculated stuffing count had been made. However, when a bacterial count was made at a temperature of 130° F., an increase of 46,000,000 was obtained. Although it is believed quite possible for bacterial increase to occur in a roasting process, the indication here is questionable. This might merely be a superficial increase attributed to slight variations in mixing procedure or sampling techniques.

It is quite prevalent at the present time for roasting procedures to designate hours cooking time or to stipulate a thigh or breast temperature to be attained.

In previous work done by Rogers and Johnson *(5)* a study of the heating characteristics of a frozen stuffed turkey was made. It was found that when the turkeys were considered done by recommended hours cooking time, the thigh muscles showed variations in temperatures ranging from 180° F. to 215° F. This is a variation of 35° F. The temperature at the center of the stuffing ranged from 169° F. to 185° F. This is a variation of 16° F. The lowest stuffing temperature was within 4 degrees of the lethal stuffing temperature of 165° F. It may be seen that a few minutes roasting time could mean the destruction or survival of food infection organisms. It is believed that an accurate measurement of the temperature at the center of the stuffing, in conjunction with a pleasing degree of doneness, is a much safer criterion than the above recommendations alone indicating roasting time or temperatures of parts of the turkey other than that at the center of the stuffing.

SUMMARY

The survival of *Salmonella pullorum* in the stuffing during the roasting of frozen stuffed turkeys has been investigated.

A temperature of 160° F. must be reached in the center of the stuffing to assure adequate destruction of *Salmonella pullorum*. To allow a modest margin of safety, a minimum temperature of 165° F. is believed to be desirable.

The roasting process of a turkey should not be interrupted until the lethal temperature of 165° F. is reached in the center of the stuffing. To partially roast a turkey and hold to complete the roasting later is not advised.

The initial temperature and the size of the turkey influence considerably the time required to reach a lethal temperature in the stuffing. The lower the initial temperature of the turkey, the longer the roasting period required.

Present recommended roasting procedures designating hours cooking time or which stipulate a thigh or breast temperature to be attained alone does not appear to be adequate bacteriologically. An accurate measurement of the temperature at the center of the stuffing, in conjunction with an attractive doneness, is believed to be a much safer criterion than the above procedures.

Acknowledgment

The writers wish to express their appreciation to Mrs. Mona Frundell for technical assistance in the performance of these experiments.

LITERATURE CITED

1. Castellani, A. G., Clarke, Ruth R., Gibson, Margaret I., and Meisner, D. F. Roasting time and temperature required to kill food poisoning microorganisms introduced experimentally into stuffing in turkeys. *Food Research*, **18**, 131-138 (1953).
2. Esselen, William B., Levine, Arthur S., and Brushway, Marion J. Adequate roasting procedures for frozen stuffed poultry. *J. Milk and Food Technology*, **19**, 196 (1956).
3. Hussemann, Dorothy L., and Wallace, Margaret A. 1951. Studies on the possibility of the transmission of Salmonella by cooked fowl. *Food Research*, **16**, 89-96 (1951).
4. Jensen, Lloyd B. *Microbiology of Meats*. 3rd ed., 1954. The Garrard Press, Champaign, Ill. p. 422.

5. ROGERS, RAYMOND E., AND JOHNSON, JOYCE. Heating characteristics of a frozen stuffed turkey in relation to the oven temperature immediately surrounding the turkey. (Unpublished).
6. *Standard Methods for the Examination of Water, Sewage, and Industrial Wastes. Public Health Assoc.*, New York, N. Y.
7. TISCHER, R. G., AND HURWICZ, H. 1954. Thermal characteristics of bacterial populations. *Food Research*, **19**, 80-91 (1954).
8. WEBSTER, R. C., AND ESSELEN, W. B. Thermal resistance of food poisoning organisms in poultry stuffing. *J. Milk and Food Technology*, **19**, 209 (1956).

ROASTING OF FROZEN STUFFED TURKEYS. II. SURVIVAL OF *MICROCOCCUS PYOGENES* VAR. *AUREUS* IN INOCULATED STUFFING

RAYMOND E. ROGERS AND MILLARD F. GUNDERSON

Research Department, Campbell Soup Company, Omaha, Nebraska

(Manuscript received July 11, 1957)

It is practically impossible to be certain that all undesirable bacteria are eliminated from food. Food handlers and cooks can contaminate food material due to the presence of organisms on their hands as well as in their respiratory tracts. Horwood *et al. (4)* found that in 30 hand washing samples obtained from food handlers, micrococci were absent in only one sample. The organisms exist in their air, on kitchen utensils, and dinner ware. Processing and preparation of some foods may serve as a means of contamination. Therefore, food should be treated in some way to destroy them, as by heating, and then held at temperatures which will prevent their growth and development.

Micrococcus pyogenes var. *aureus* cannot be commonly classed as a heat-resistant organism because it is not a spore former. In comparison with other nonspore-forming bacteria, however, it is more resistant than many of them. Dack *et al. (2)* reported that a strain isolated from sponge cakes, which had been incriminated in food poisoning, was killed at 176° F. in 15 minutes. McBurney *(7)* also made such observations. As many as 2,500,000 micrococci per ml. were completely destroyed by the cooking process, which was carried out in a bakery at 185° F. for 10 minutes.

Saslow, Scherago, and Weaver *(9)* isolated a strain from oysters which showed a resistance of 10 minutes at 194° F. One strain of *Micrococcus pyogenes* var. *aureus* studied by Hassemann and Tanner *(5)* survived 30 minutes at 131° F. and was destroyed in less than 3 minutes at 185° F. Thermal resistance studies by Webster and Esselen *(12)* of *Micrococcus pyogenes* var. *aureus* in poultry stuffing showed that the organisms survived 8 minutes at 140° F. when the initial concentration of organisms in the stuffing was 50,000 to 100,000 organisms per ml.

It was the purpose of this investigation to determine the temperature treatment required to render a stuffing free from *Micrococcus pyogenes* var. *aureus*.

EXPERIMENTAL PROCEDURE

Culture. The University of Nebraska Medical School provided the culture of *Micrococcus pyogenes* var. *aureus* used in this study. No previous history of this culture was accessible. Brain heart infusion agar slants served to carry the culture which was transferred bi-weekly. The culture was transferred and incubated for 24 hours at 37° C. 48 hours previous to the inoculation of the stuffing. Twenty-four hours prior to the inoculation of the stuffing a transfer was made from the slants to brain heart infusion broth and incubated at 37° C. This broth culture was used to inoculate the stuffing. For both the medium and the large size birds a new batch of inoculum was prepared for the inoculation of the stuffing employed.

Inoculation and stuffing. Six frozen unstuffed turkeys with weights ranging from 10.1 to 10.6 pounds, and six with weights ranging from 17.15 to 20.2 pounds were placed in watertight plastic bags, sealed, and submerged in water to thaw. The turkeys were removed from the water after thawing, the plastic bags discarded, and the giblets removed from the body cavity. The weight of each turkey was determined. The body cavities were closed by means of metal skewers after stuffing. The birds were again

weighed and the quantity of stuffing each contained calculated by difference. After insertion into plastic bags and sealed the turkeys were immediately placed in a −40° F. to −50° F. wind tunnel. The turkeys were removed when completely frozen and stored in a freezer with a temperature of −9.4° F. The turkeys remained in storage from 2 to 10 days before testing. The number of *Micrococcus pyogenes* var. *aureus* was not determined immediately prior to roasting. Straka and Combes *(10)* studied the survival of *Micrococcus pyogenes* var. *aureus* in creamed chicken under various storage conditions. Inoculated containers of creamed chicken were placed in a −30° F. freezer immediately after the cans were sealed. Containers when sampled were defrosted immediately in warm water. The count did not vary a great deal during storage of 1 year. In this investigation the number of organisms was presumed to be the same as at the time of inoculation.

On duplicate 11 g. samples of frozen uninoculated commercial stuffing a total bacterial count was made. The stuffing was placed in a large pan, broken up, and mixed by hand with a 24-hour brain heart infusion culture of *Micrococcus pyogenes* var. *aureus*. The inoculum was dispensed at the approximate concentration of 15 ml. of culture for each 50 g. of stuffing inoculated. To determine the number of *Micrococcus pyogenes* var. *aureus* organisms per gram of stuffing duplicate 11 g. samples of the inoculated stuffing were tested.

Roasting procedure. The plastic bag was discarded and the turkey was placed on a metal roasting pan as each was prepared for testing. An electric drill was employed to drill holes into the turkey at an angle so that when the copper constantan thermocouples were inserted, the pips would be in the approximate geometric center of the stuffing. From preliminary studies done by Rogers and Johnson *(8)* on the heating characteristics of frozen stuffed turkeys, the center of the stuffing was found to heat the least rapidly. A reading was made previous to placing the turkey in the oven after waiting for a period of 5 minutes to allow the thermocouples to equilibrate with the stuffing temperature.

Two thermocouples, previously placed in the oven, were both in the approximate center of the oven one close to the top of the turkey, the other close to the bottom of the turkey (See Figure 1, *8a*). A Leeds and Northrup potentiometer with a calibrated temperature scale was used to determine the temperature of the oven and the center of the stuffing before and throughout the roasting process (See Figure 2, *8a*). During the roasting of the medium-size turkeys a reading was made every 10 minutes and during the roasting of the large-size turkeys every 15 minutes. At a designated temperature for testing and analyzing each turkey was removed from the oven.

Bacteriological methods. Total bacterial counts were made by plating on tryptone glucose extract agar. Duplicate 11 g. samples of frozen uninoculated stuffing were macerated by a Waring blendor in 99 ml. of sterile distilled water. Plates were incubated at 37° C. for 24 hours.

The pour-plate technique was employed to determine the number of *Micrococcus pyogenes* var. *aureus* organisms per gram of the inoculated, unroasted and roasted stuffing. Mannitol salt agar was used as the plating medium. Plates were incubated for 24 hours at 37° C.

RESULTS AND DISCUSSION

The results of Table 1 show the relationship of time and temperature required to kill *Micrococcus pyogenes* var. *aureus* during the roasting of medium-size frozen stuffed turkeys. With the stuffing possessing an initial count of 67,000,000 organisms per gram, a minimum temperature of 165° F. must be reached in the center of the stuffing of the medium-size turkeys to assure adequate destruction of the organisms. Ninety-seven thousand micrococci per gram still survived when the stuffing temperature was 160° F. It required a roasting period of 4 hours and 50 minutes to reach a lethal temperature when the initial temperature of the stuffing was 0° F.

The time and temperature required to kill *Micrococcus pyogenes* var. *aureus* during the roasting of large frozen stuffed turkeys is shown in the results of

TABLE 1

Survival of *Micrococcus pyogenes* var. *aureus* in stuffing of roasted, medium size, frozen stuffed turkeys[1]

Turkey No.	Weight (lbs) of Stuffed Bird	Weight (lbs) of Stuffing	Initial Temp. (° F.) of Stuffing	Roasting time at 325° F.	Highest ° F. Temp. of Cent. of Stuffing	Survival of *Micrococcus*/gm of roasted stuffing
1	10.60	2.13	5	3 hrs. 40 min.	100	84,000,000
8	10.40	2.12	0	4 hrs. 5 min.	135	90,000,000
4	10.10	2.13	0	4 hrs. 10 min.	145	700,000
2	10.12	3.30	8	4 hrs. 25 min.	150	200,000
3	10.13	3.30	0	4 hrs. 50 min.	160	97,000
7	10.40	2.11	0	4 hrs. 10 min.	165	0

[1] Initial count of inoculated stuffing was 67,000,000 organisms per gram of stuffing.

TABLE 2

Survival of *Micrococcus pyogenes* var. *aureus* in stuffing of roasted, large size, frozen stuffed turkeys[1]

Turkey No.	Weight (lbs) of Stuffed Bird	Weight (lbs) of Stuffing	Initial Temp. (° F.) of Stuffing	Roasting time at 325° F.	Highest ° F. Temp. of Cent. of Stuffing	Survival of *Micrococcus*/gm of roasted stuffing
2	20.20	5.10	0	5 hrs. 30 min.	100	121,000,000
3	18.40	4.50	0	6 hrs. 30 min.	140	1,400,000
4	18.20	4.70	0	6 hrs. 30 min.	145	55,000
7	18.50	3.10	0	6 hrs. 45 min.	155	1,000
5	17.15	4.00	0	7 hrs. 15 min.	160	0
8	18.10	3.30	0	7 hrs. 15 min.	165	0

[1] Initial count of inoculated stuffing was 149,000,000 organisms per gram of stuffing.

Table 2. A minimum temperature of 160° F. must be reached in the center of the stuffing of the large turkeys for the complete destruction of the organisms. With the initial sample of stuffing possessing 149,000,000 organisms per gram, at a temperature of 155° F., 1,000 organisms per gram still survived. A roasting period of 7 hours and 15 minutes was required to reach a lethal temperature of 160° F. in the center of the stuffing when the initial temperature was 0° F.

Because of the slower rate of heat penetration of the larger birds (12 to 15 pounds) Esselen *et al. (3)* found that they required a longer cooking time. Before removing the bird from the oven they recommended that a center temperature of 155° F. be attained. For cooking frozen birds their recommendations required no special precautions other than to roast to what is considered to be a palatable and attractive degree of doneness and a center temperature as indicated for the different weight classes. A temperature of 165° F. reached at the center of the stuffing during the roasting period, Castellani *et al. (1)* found, appeared sufficient to kill *Micrococcus pyogenes* var. *aureus* and allow a modest margin of safety. In no instance did the stuffing of the turkeys yield initial counts exceedng 5,000,000 organisms per gram. In one experiment the inoculated stuffing possessed a total bacterial count of 4,019,000 organisms per gram, of which 2,513,000 were micrococci. When a sample was removed from the center of the stuffing after a temperature of 125° F. had been attained, it was found that *Micrococcus pyogenes* var. *aureus* still survived. In another experiment the inoculated stuffing possessed a total bacterial count of 1,530,000 organisms per gram, of which 830,000 were *Micrococcus pyogenes* var. *aureus*. A temperature of 154° F. at the center of the stuffing was sufficient to destroy the micrococci. A more drastic heat treatment is required to destroy a large number of cells than a few *(11)*. This is shown in the results of Table 1 and 2 when a temperature of 160° F. and 165° F. respectively was necessary to destroy all the micrococci in stuffing which yielded initial counts of 149,000,000 and 67,000,000 organisms per gram. These observations are important in food preservation, for this is the basis for efforts to keep the number of bacteria in food low.

The roasting process of a turkey should not be interrupted. As shown in the results of Table 1, *Micrococcus pyogenes* var. *aureus* survived a temperature of 160° F. when the stuffing had a bacterial count of 97,000 organisms per gram. In Table 2 *Micrococcus pyogenes* var. *aureus* survived a temperature of 155° F. when the stuffing had a bacterial count of 1,000 organisms per gram. Jensen *(6)* found that food remaining between the temperatures of 50° F. and 118.4° F. for a period of from 4 to 8 hours was potentially dangerous. Partially roasting a turkey would place the bird in this danger zone not once, but 3 times: (a) during the initial roasting; (b) during the initial cooling; and (c) during the second roasting.

Figure 1 shows graphically the changes in the bacterial population of *Micrococcus pyogenes* var. *aureus* in the stuffing during the roasting of medium and large frozen turkeys. From an initial stuffing count of 67,000,000 organisms per gram in the medium size turkeys at a roasting temperature of 100° F. the bacterial count had increased to 84,000,000 organisms per gram. When a bacterial count was made at a temperature of 135° F. an increase to 90,000,000 organisms per gram was obtained. Although it is believed quite possible for bacterial multiplication to occur in stuffing during the roasting process, the

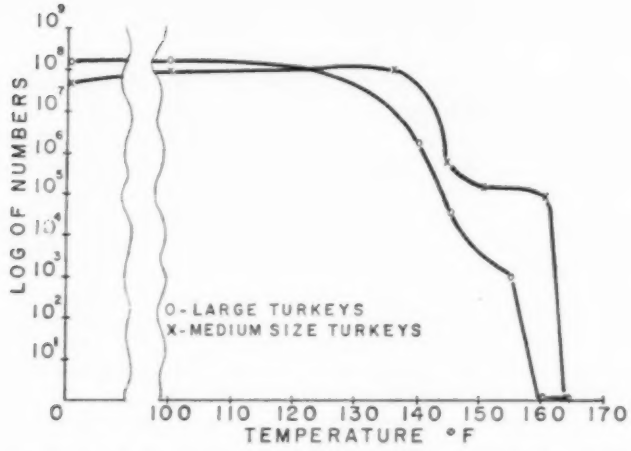


Figure 1. Survival of micrococcus pyogenes var. aureus in the stuffing during the roasting of medium and large size frozen stuffed turkeys.

indication here is questionable. This increase might merely be superficial, attributed to slight variations in mixing procedure or sampling technique. From an initial stuffing count of 149,000,000 organisms per gram in the roasting of the large turkeys, at a roasting temperature of 100° F. the bacterial count had decreased to 121,000,000 organisms per gram. This temperature range is not believed to be lethal as it would seem indicated. This decrease might possibly be due to the fact that a bacterial count was not made on the stuffing immediately prior to the roasting of the turkeys. A decrease in the number of micrococci might have occurred in the process of freezing and storage after the initial inoculated stuffing count had been made. Again, as mentioned above, this also might be attributed to mixing procedures or sampling technique.

Roasting procedures, at the present time, designating hours cooking time or stipulating a thigh or breast temperature to be attained is prevalent. A study of the heating characteristics of a frozen stuffed turkey was previously made by Rogers and Johnson *(8)*. When the turkeys were considered done by recommended hours of cooking time, it was found that the thigh muscles showed variations in temperatures ranging from 180° F. to 215° F. This is a variation of 35° F. The temperature at the center of the stuffing ranged from 169° F. to 185° F. This is a variation of 16° F. The lowest stuffing temperature was within 4 degrees of the lethal stuffing temperature of 165° F. Depending on the initial temperature and size of the turkey, a few minutes roasting time could mean a considerable variation in the roasting temperature attained. This could mean the destruction or survival of food poisoning organisms. It is believed that an accurate measurement of the temperature at the center of the stuffing, along with the attainment of an attractive doneness, would be a much safer criterion than the above recommendations alone indicating roasting time or temperatures of parts of the turkey to be attained other than that at the

center of the stuffing. Thus, the bacteriological safety of the product to be consumed would be assured.

SUMMARY

The survival of *Micrococcus pyogenes* var. *aureus* in the stuffing of frozen turkeys has been investigated wtih the following observations:

A minimum temperature of 165° F. must be reached in the center of the stuffing during the roasting period to assure adequate destruction of *Micrococcus pyogenes* var. *aureus*. This, however, allows little margin of safety.

Micrococcus pyogenes var. *aureus* survived a temperature of 160° F. in the center of the stuffing. Therefore, the roasting process should not be interrupted until a lethal temperature of 165° F. has been reached in the center of the stuffing.

Roasting procedures designating hours cooking time or stipulating a thigh or breast temperature to be attained alone, again as in the preceding experiment, does not appear to be reliably safe bacteriologically. A sound procedure such as the determination of the temperature at the center of the stuffing, complimented by the attainment of a pleasing appearance of doneness, would be a much safer criterion than other recommended roasting procedures.

Acknowledgment

The writers wish to express their appreciation to Mrs. Mona Frundell for technical assistance in the performance of these experiments.

LITERATURE CITED

1. Castellani, A. G., Clarke, Ruth R., Gibson, Margaret I., and Meisner, D. F. Roasting time and temperature required to kill food poisoning microorganisms introduced experimentally into stuffing in turkeys. *Food Research*, **18**, 131-138 (1953).
2. Dack, Gail, *et al.* An outbreak of food poisoning proved to be a yellow hemolytic *Staphylococcus*. *J. Prev. Med.*, **4**, 167-175 (1930).
3. Esselen, William B., Levine, Arthur S., and Brushwya, Marion J. Adequate roasting procedures for frozen stuffed poultry. *J. Milk and Food Technology*, **19**, 196 (1956).
4. Horwood, Murray P., and Minch, Virgil A. The numbers and types of bacteria found on the hands of food handlers. *Food Research*, **16**, 133-136 (1951).
5. Hussemann, D. L., and Tanner, Fred W. Relation of certain cooking precedures to *Staphylococcus* food poisoning. *Am. J. Pub. Health*, **37**, 1407-1414 (1947).
6. Jensen, Lloyd B. *Microbiology of Meats*. 3rd ed., 1954. The Garrard Press, Champaign, Ill. pp. 422.
7. McBurney, F. Food Poisoning due to *Staphylococci*. *J. Am. Med. Assoc.*, **100**, 1999-2001 (1933).
8. Rogers, Raymond E., and Johnson, Joyce. Heating characteristics of a frozen stuffed turkey in relation to the oven temperature immediately surrounding the turkey. (Unpublished).
8a. Rogers, Raymond E., and Gunderson, Millard F. Roasting of frozen stuffed turkeys. I. Survival of *Salmonella pullorum* in inoculated stuffing. *Food Research*. (This issue.)
9. Saslaw, Samuel, Scherago, M., and Weaver, R. H. Heat resistance and possible spore formation in a food poisoning *Staphylococcus*. *J. Bacteriol.*, **39**, 16 (1940).
10. Straka, Robert P., and Combes, Frances M. 1952. Survival and multiplication of *Micrococcus pyogenes* var. *aureus* in cream chicken under various holding, storage and defrosting conditions. *Food Research*, **17**, 448-445 (1952).
11. Tischer, R. G., and Hurwicz, H. Thermal characteristics of bacterial population. *Food Research*, **10**, 80-91 (1954).
12. Webster, R. C., and Esselen, W. B. 1956. Thermal resistance of food poisoning organisms in poultry stuffing. *J. Milk and Food Technology*, **19**, 209 (1956).

VOLATILE CARBONYL COMPOUNDS OF COOKED CHICKEN. I. COMPOUNDS OBTAINED BY AIR ENTRAINMENT

E. L. PIPPEN, M. NONAKA, F. T. JONES, AND FRED STITT

Western Regional Research Laboratory, Albany, Calif.*

(Manuscript received July 25, 1957)

Progress toward production of poultry products having stable and optimum flavor depends considerably on obtaining increased insight into the fundamental causes of flavor and off flavor of the products. To provide part of this basic information, studies on composition of the volatile fraction of cooked chicken have been in progress at this laboratory. A previous paper *(12)* reported properties of volatile sulfur and nitrogen compounds. The investigation reported here describes a method of isolating volatile carbonyl compounds from cooked chicken, as the 2,4-dinitrophenylhydrazone derivatives, and the subsequent chromatographic separation of the mixed hydrazones into fractions which enabled identification of many of the carbonyl compounds.

EXPERIMENTAL

Isolation of volatile carbonyl compounds from cooked chicken. Throughout the study, 2,4-dinitrophenylhydrazine (2 g. per liter of 2 *N* HCl) reagent was used to detect presence of carbonyl compounds. Three sets of conditions for isolating volatile carbonyl compounds were considered. These comprised recovery of volatiles evolved from chicken cooked under (a) normal cooking conditions, (b) oxidation-inhibiting conditions, and (c) oxidation-favoring conditions. For condition (a), chickens were simmered in water for 3 hours and distillate of the resulting broth was tested for presence of carbonyl compounds. For conditions (b) and (c), a stream of nitrogen and air, respectively, were passed through each system during cooking, not only to provide an inert and oxidative atmosphere, but also to entrain and sweep volatile components into the detecting reagent. A volatile carbonyl fraction was detected for all 3 isolation methods. Preliminary chromatography of the hydrazones on silicic acid-Celite columns, as described below, showed that regardless of the isolation method a complex mixture of hydrazones was obtained. Of the 3 methods, the one favoring oxidation gave the greatest yield of carbonyl while the one utilizing a nitrogen atmosphere gave the lowest yield. In some respects method (a), based on a normal cooking procedure and utilizing chicken broth distillate, which has been shown *(12)* to contain detectable flavor, would be a logical starting point for this investigation. However it was decided to study the qualitative nature of the carbonyl fraction isolated by both methods (a) and (c). Such a comparison would show whether a qualitative as well as a quantitative difference exists in the carbonyl fraction isolated and might provide some clues about the nature of carbonyl compounds which are associated with both normal and oxidative off flavors. It was decided to work first on carbonyl compounds isolated by the atypical oxidation-favoring cooking conditions described below, because this method provided a yield of carbonyl compounds sufficient to make identification of minor constituents seem feasible.

The apparatus used for isolation of carbonyl compounds as 2,4-dinitrophenylhydrazones is shown in Figure 1.

---

* Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

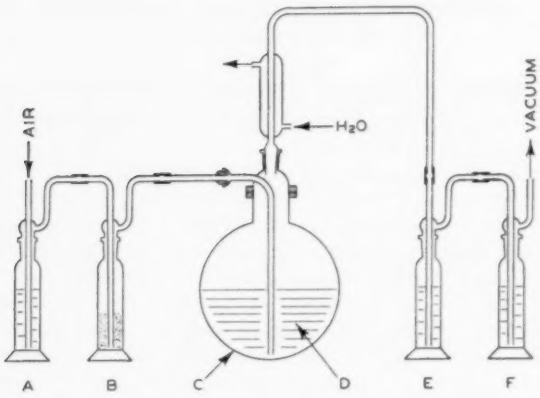


Figure 1. Apparatus used to isolate volatile carbonyl compounds from chicken.

A.—400 ml. of a saturated solution of 2,4-dinitrophenylhydrazine in 2*N* sulfuric acid.
B.—Trap containing glass wool.
C.—22 liter flask.
D.—Chicken and water heated to slow boil.
E. and F.—400 ml. of 2,4-dinitrophenylhydrazine solution (2g/liter in 2*N* HCl).

Three ready-to-cook chicken carcasses were used for each run. Each carcass was separated into 8 parts. Gross deposits of visceral fat were removed. However, a generous amount of fat remained in the form of subcutaneous and intra-muscular deposits. The weighed chicken parts and an equal weight of distilled water were placed in flask C. After closure of the flask, air was drawn through the system by applying enough vacuum at trap F to cause approximately 2 bubbles per second in traps E and F. Then, with water circulating through the condenser, sufficient heat was applied to the flask containing chicken to maintain gentle refluxing throughout the run. The duration of a run was calculated from start of refluxing to the time when heating was stopped. Each run extended over a period of approximately 20 hours. At the end of a run, hydrazones in traps E and F were filtered off, washed with water and dried *in vacuo* at 60° C. From 7 such runs, 7. 8 g. of 2, 4-dinitrophenylhydrazones were collected from a total of 31.4 kg. of chicken.

Separation of hydrazones into mono and polycarbonyl fractions. Before attempting separation of the mixed hydrazones obtained from the cooked chicken, it was found convenient and desirable to fractionate it first into mono- and polycarbonyl components. To accomplish this, advantage was taken of the extreme insolubility of the 2,4-dinitrophenylhydrazone derivatives of polycarbonyl compounds *(8)*. The latter are only slightly soluble in hot alcohol or chloroform while the monocarbonyl derivatives are quite soluble in these solvents. Accordingly the hydrazones were treated on a filter with generous amounts of hot alcohol and chloroform. The portion appearing in the filtrate was recovered by evaporating the solvent on a steam bath in a stream of nitrogen. It constituted the major portion of the hydrazone fraction, gave a red color in alcoholic KOH characteristic of mono-2,4-dinitrophenylhydrazone derivatives *(11)*, and was designated the mono-2,4-dinitrophenylhydrazone fraction. The insoluble derivatives remaining on the filter gave a violet color in alcoholic KOH characteristic of bis-2,4-dinitrophenylhydrazone derivatives of compounds having adjacent dicarbonyl groups *(11)*. It was designated the poly carbonyl fraction. Fractionation of 6.3 g. of hydrazone, isolated from chicken as described above, gave 5.8 g. of the monocarbonyl fraction and 0.5 g. of the polycarbonyl fraction.

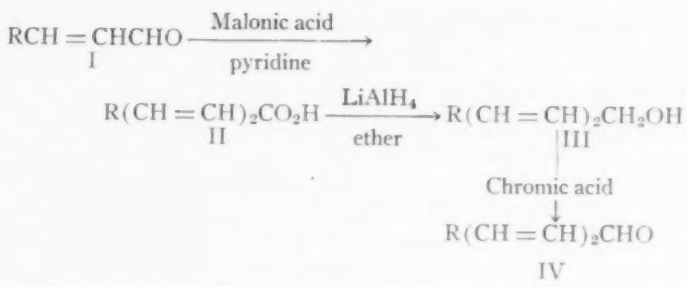
Isolation of diacetyl 2,4-dinitrophenylhydrazone. The polycarbonyl 2,4-dinitrophenylhydrazone fraction was taken up in boiling nitrobenzene, filtered and set aside at room temperature. After several days, crystals which had separated were filtered off, washed generously with alcohol and dried *in vacuo* at 100° C. for 16 hours. Comparison of the melting point, optical properties and X-ray diffraction pattern of these crystals with those of authentic diacetyl bis-2,4-dinitrophenylhydrazone showed that they were indentical. Results of ultimate analysis (Table 2) confirm this conclusion.

Chromatography of the monocarbonyl 2,4-dinitrophenylhydrazone fraction. Chromatography was carried out in glass columns (35 mm. outside diameter). The adsorbent (silicic acid-Celite, 2 to 1 weight ratio), developing solvents and general procedure used were those of Gordon, Wopat, Burnham and Jones *(6)*. The petroleum ether, b.p. 30-60° C., was purified by treating it successively with conc. sulfuric acid, water, 3*N* KOH, and water. It was then dried over anhydrous calcium sulfate, and distilled. The fraction boiling from 40 to 50° C. was used. Ether and chloroform were also redistilled. A preliminary separation of 5.8 g. of the monocarbonyl-2,4-dinitrophenylhydrazone fraction was accomplished by chromatographing it in 100 to 200 mg. lots on 33 columns (each packed to a 9-inch height). This gave 8 preliminary fractions with the weights shown in Fig. 2. Similar fractions were combined and rechromatographed mostly through adsorbent packed to a 9-inch height but occasionally through 18 and 30 inch heights after it was discovered that additional length permitted better separation in some instances. The first repass sometimes provided fractions of sufficient purity to permit identification after crystallization from ethanol. For more difficult cases crystallization and chromatography were repeated until the procedure no longer improved the purity of the fraction. In many instances, even though complete separation was not evident, it was possible to obtain a portion of the components in a pure state by taking many small fractions of a broad band as it eluted from the column. After collecting a fraction it was evaporated to dryness on a steam bath with the aid of a nitrogen stream. The residue was transferred with chloroform to a weighed flask. After evaporation of solvent, the weight of the residue was recorded. Following this, the fraction was crystallized from ethanol and, if sufficiently pure, characterized. Yield of a particular hydrazone was based on the weight of the fraction as obtained from the column and from which the pure crystalline hydrazone was subsequently obtained.

Paper chromatography of 2,4-dinitrophenylhydrazones. Paper chromatography, as an aid to identification of 2,4-dinitrophenylhydrazone derivatives isolated from chicken, was carried out as described by Lynn, Steele, and Staple *(7)*. Whatman No. 1 filter paper was used.

Melting point determinations. Melting points were determined with the sample between cover glasses on a commercially available electrically heated stage which had been calibrated with a set of test reagents. Hence melting points reported here may be considered corrected. A microscope was mounted above the stage to provide good visibility.

Preparation of authentic 2,4-dinitrophenylhydrazone derivatives. The carbonyl compound was added to a solution of 2,4-dinitrophenylhydrazine (2 g./liter in 2 *N* HCL). A 10% excess of 2,4-dinitrophenylhydrazine reagent was used. If the carbonyl compound was immiscible with the 2,4-dinitrophenylhydrazine solution, the mixture was agitated and warmed on a steam bath. The hydrazone was filtered off and washed with water. For monocarbonyl derivatives this preparation was followed by recrystallization from ethanol, chromatography on silicic acid-Celite columns to remove impurities, and crystallization of the purified product from ethanol. In several instances this method of purification resulted in derivatives melting considerably higher than values reported in the literature. Polycarbonyl 2,4-dinitrophenylhydrazone derivatives were purified by extracting crude preparation with hot ethanol (to remove any soluble monocarbonyl derivative) followed by several recrystallizations from nitrobenzene. After the final crystallization crystals were washed with alcohol and dried overnight *in vacuo* at 100° C. to remove traces of nitrobenzene. The n-aliphatic-2-en-1-als which were not commercially or otherwise available, were synthesized as described by Martin, Schepartz, and Daubert *(9)*. The six and seven carbon n-aliphatic-2,4-dien-1-als which were available for this study were prepared according to the following scheme:



Condensation of the 2-enals, I, with malonic acid in pyridine gave about 30% yields of the 2,4-dienoic acids, II. The latter were reduced to the corresponding alcohols, III, in yields of the order of 60%. However, attempts to convert the dienols, III, to the aldehydes, IV, by low temperature chromic acid oxidation *(3)*, gave very low yields (0 to 2%) of the desired aldehyde, which was mixed with other carbonyl compounds arising from cleavage of the carbon chain, and hence difficult to purify. The crude aldehydes, IV, were converted to the 2,4-dinitrophenylhydrazones and the latter were purified by chromatography on silicic acid-Celite columns. Because of the low yield and purification difficulties, it was decided that preparation of further members of the series should await improvements in the synthetic procedure. Two crystalline forms of the 2,4-dinitrophenylhydrazone of n-hepta-2,4-dien-1-al were obtained. One form, m.p. 160.5 to 162.5° C., consisted of plates which appeared brown under ordinary light and deep red under strong transmitted light. The other form, m.p. 155 to 159° C., consisted of purple-red needles. Other authentic carbonyl compounds required were obtained from commerical sources.

Source and processing of chickens. Fowl used in this study were purchased in either a packaged, frozen, cut-up, ready-to-cook form or in the live state from commercial sources. The latter were processed to the ready-to-cook point and packaged in polyethylene at this laboratory. Fowl were held at -10° F. until used.

Microscopy. All fractions were examined microscopically. Observations of optical and crystallographic properties were made with a polarizing microscope. The isolated, purified 2,4-dinitrophenylhydrazone fractions were compared with authentic 2,4-dinitrophenylhydrazones by melting a portion of the unknown under a cover glass in such a way that not all of the space was filled by the sample. After freezing, the remaining space was filled with the melt of an authentic 2,4-dinitrophenylhydrazone, suspected to be the same as the unknown. After the latter had frozen, the pair was remelted across the zone of contact by holding a hot cautery needle close to the cover glass. This permitted observation of melting point differences, melting point depression, eutectic formation, solid solution formation, phase transformation, crystallization habit, sublimation, rates of crystallization, and color differences. Crystals grown from the melt could be tested for dichroism, extinction angle, optic orientation and dispersion of interference figures, and, if necessary, refractive indices. Since this method permits unequivocal identification and requires only micro amounts of material, it proved to be the most satisfactory method available for confirming the identity of several of the smaller fractions. It is well known that many of the 2,4-dinitrophenylhydrazones exist in various polymorphic forms which may exhibit different melting points *(2)*. These forms lead to confusion unless they are recognized. The type of microscopic examination mentioned above proved very helpful as a means of recognizing various polymorphic forms, and often transformation from one form to another could be observed during the microscopic examination.

Absorption spectra. Ultraviolet spectra of chloroform solutions of the 2,4-dinitrophenylhydrazones were obtained with a Model 14 Cary recording spectrophotometer. Infrared spectra of samples in the form of potassium bromide disks were obtained from 2 to 15 μ with a Model IR-3 Beckman spectrophotometer using sodium chloride

prisms.[b] A standardized procedure for the preparation of samples was employed in which the 2,4-dinitrophenylhydrazone was dissolved in a few drops of chloroform, hand-ground in a small mortar with potassium bromide powder, the mixture evacuated and pressed *in vacuo*. Although this procedure was designed to yield spectra which are independent of the particular polymorphic forms of the original samples, examples have been encountered which indicate that this procedure cannot always be relied upon for this purpose.

RESULTS

Using the air entrainment method, as described in the experimental section, 7.8 g. of 2,4-dinitrophenylhydrazone precipitate was obtained from 31.4 kg. of chicken. The general method of fractionation of this hydrazone precipitate into the several purified hydrazones is shown schematically in Fig. 2.

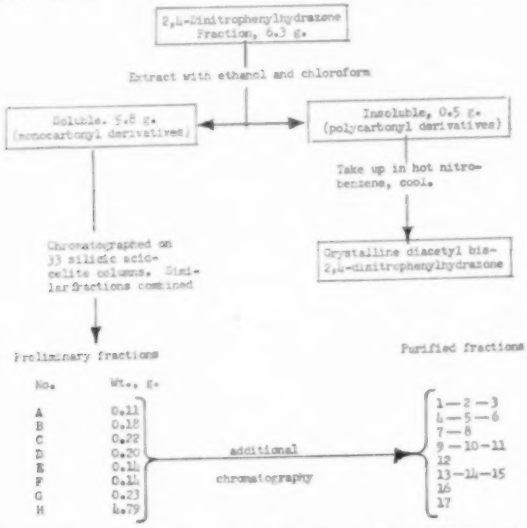


Figure 2. Fractionation scheme used for separating 2,4-dinitrophenylhydrazones of carbonyl compounds isolated from cooked chicken.

Treatment of the hydrazone precipitate with solvents proved an effective way of separating the bulk of the mono- from the poly carbonyl derivatives and offered a convenient method for isolating the pure derivative of diacetyl. Inspection of the data in Table 1 and 2 shows that diacetyl-bis-2,4-dinitrophenylhydrazone was obtained by direct crystallization from a nitrobenzene solution of the polycarbonyl derivatives. Hence it seems reasonable to assume that most of the polycarbonyl 2,4-dinitrophenylhydrazone fraction consisted of the diacetyl derivative. On this basis, this diketone ranks second in abundance among the carbonyl compounds isolated. However during chromatography of the monocarbonyl derivatives there was some evidence of the presence of other polycarbonyl derivatives. This evidence was in the form of several small bands

[b] Mention of a commercial name does not mean that it is recommended by the Department of Agriculture over similar products not mentioned.

running after acetaldehyde-2,4-dinitrophenylhydrazone on the column and which, when treated with alkali in ethanol, gave a blue-violet color characteristic of bis-2,4-dinitrophenylhydrazones of compounds having carbonyl groups on adjacent carbon atoms *(11)*. Doubtless these bands can be attributed to solubilization of a small amount of polycarbonyl 2,4-dinitrophenylhydrazone during the chloroform-alcohol fractionation step thus resulting in their appearance on the column with the monocarbonyl derivatives. The amount of material these bands represented was very small and they were not investigated further.

A single passage of the monocarbonyl derivatives through 9-inch beds of adsorbents gave the 8 preliminary fractions with yields shown in Fig. 2 Fraction A moved fastest; fraction H, slowest. Of these, only fraction H (acetaldehyde-2,4-dinitrophenylhydrazone) proved to be pure. The yield of this fraction shows that it was by far the most abundant carbonyl derivative isolated. Other preliminary fractions when readsorbed and again chromatographed showed the presence of more than one component. Chromatography of these fractions was repeated until no further purification could be observed or until the size of the fraction became too small to permit futher manipulation. In Fig. 2 under "purified fractions" are listed the numbers of the fractions which were resolved in a sufficiently pure state to permit partial or complete identification. From this figure it is apparent that preliminary fraction A yielded 3 purified fractions (1, 2 and 3), B yielded 3 purified fractions (4, 5 and 6), etc. Hence the purified fractions are listed in the actual order of their movement down the column and this order of listing is carried over into Table 1.

It is clear from an inspection of the yields in Table 1 that not all of the hydrazone encountered in the preliminary fractions is accounted for as purified material. Excluding acetaldehyde 2,4-dinitrophenylhydrazone (which is entirely accounted for in preliminary fraction H) purified fractions 1 through 16 account for 60% of the hydrazone present in preliminary fractions, A through G. Most of the material not accounted for probably represents mixtures of hydrazones identified in this study which were obtained as fore-runs and after-runs of a necessarily small fraction taken in cases of difficult separation to help insure obtaining a sample of sufficient purity to permit characterization. On the other hand, an appreciable portion of the hydrazone not accounted for doubtless represents unidentified derivatives which were not resolved. This is particularly true for preliminary fractions E and G from which only 1.4% and 20%, respectively, of purified material was obtained.

As indicated in Tables 1 and 2, 15 of the 17 monocarbonyl derivative fractions isolated chromatographically were identified by comparison with authentic derivatives with respect to 3 or more of the following criteria: melting point, elemental composition, chromatographic behavior, crystallographic properties, ultraviolet and infrared absorption spectra. Except for acetone and methyl ethyl ketone, all of these are derivatives of aldehydes, namely, the n-alkan-1-als containing 2, 3, 4, 5, 6, 8, and 9 carbon atoms, the n-alk-2-en-1-als containing 5, 6, 7, 10, and 11 carbon atoms, and n-hepta-2,4-dien-1-al.

Identification of fraction 14 (Table 1) as a mixture of the 2,4-dinitrophenylhyrdazone derivatives of n-butanal and methyl-ethyl-ketone seems open

TABLE 1

Properties of 2,4-dinitrophenylhydrazones of carbonyl compounds isolated from cooked chicken

Fraction Number[1]	Weight mg.	Recrystallized fraction M.p., °C	Carbonyl compound identified	Authentic sample M.p., °C	Mixed with authentic sample, M.p., °C	Additional methods of characterization
1	7	122.5-123.8	n-undec-2-en-1-al	122 -123.5	122 -123.5	2, 4, 10
2	53	125 -126	n-dec-2-en-1-al	127 -128.5	125 -128	2, 5, 10
3	15	141 -142.5	2,4-dien-1-al	unavailable	.....	3
4	15	102 -106	n-nonanal	108 -108.6	103 -107	5, 10
5	28	102 -107	n-octanal	107.5-108.4	102 -107	5, 10
6	77	131 -138	2,4-dien-1-al	unavailable	.....	3, 6
7	108	130 -133	n-hept-2-en-1-al	133.7-134.5	130 -133	2, 4, 6, 10
8	254	105 -108	n-hexanal	108.5-109.7	105 -108	6, 10
9	17	147.8-149.6	n-hex-2-en-1-al	148.5-149	148 -149	4, 10
10	42	108.5-110	n-pentanal	108.5-110.5	108.5-110	4, 10
11	24	156 -162	n-hepta-2,4,-dien-1-al	160.5-162.5	155 -160	3, 4, 10
12	2	159 -161.5	n-pent-2-en-1-al	159 -161.5	159 -161.5	10, 11
13	9	119.5-122.5	n-butanal	123 -124	120 -123	5, 10
14	23	104 -112	Mixture of methyl-ethyl -ketone and n-butanal	.....	.....	7, 8, 10
15	17	148 -153.7	propanal	155 -156.7	148.5-154	5, 10
16	47	123.5-126.7	acetone	125 -127	124 -127	6, 10
17	4790	167 -168	acetaldehyde	167.5-169	167 -168	4, 6, 10
poly-carbonyl	500	d. > 300	diacetyl	d. > 300	d. > 300	6, 9, 10

[1] Fractions are numbered in the order of their relative speed of movement on the column. Fraction No. 1 = fastest running; No. 17 = slowest.

[2] Shows ultra violet absorption characteristic of 2-en-1-al derivatives: λ max. = 375 $m\mu$; ϵ max. = 29,000.

[3] Shows ultra violet absorption characteristic of 2,4-dien-1-al derivatives: λ max. = 389 $m\mu$.

[4] The infra-red spectrum was identical to that of the authentic sample.

[5] The infra-red spectrum was identical to that of the authentic sample except for very minor differences.

[6] Elemental analysis. See Table II.

[7] The infra-red spectrum of this fraction was compatible with its being a mixture of the n-butanal and methyl-ethyl ketone derivatives. Separate crystals of the two compounds were picked out under a microscope and identified microscopically.

[8] Migration on paper chromatograms was checked with authentic samples.

[9] The X-ray diffraction pattern of the crystalline isolated material was identical to that of authentic diacetyl-bis-2,4-dinitrophenylhydrazone except for very minor differences.

[10] Examination of crystals of the unknown and of the authentic sample with a polarizing microscope showed that the two were alike with respect to two or more properties such as refractive indices, phase transformations, etc.

[11] The infra-red spectrum was too weak for reliable comparison with that of the authentic material, but was compatible with the latter.

TABLE 2

Analysis of purified 2,4-dinitrophenylhydrazone fractions

Fraction Number[1]	Carbonyl Compound and Formula of Hydrazone	Carbon, % Calc'd.	Carbon, % Found	Hydrogen, % Calc'd.	Hydrogen, % Found	Nitrogen, % Calc'd.	Nitrogen, % Found
6	n-deca-2, 4-dien-1-al $C_{16}H_{20}O_4N_4$	57.82	57.3	6.07	6.2	16.86	[2, 3]
7	n-hepta-2-en-1-al $C_{13}H_{16}O_4N_4$	53.42	53.4	5.52	5.5	19.17	[3]
8	n-hexanal $C_{12}H_{16}O_4N_4$	51.42	51.6	5.75	5.6	19.99	19.5
16	acetone $C_9H_{10}O_4N_4$	45.38	45.4	4.23	4.1	23.52	[3]
17	acetaldehyde $C_8H_8O_4N_4$	42.86	42.9	3.60	3.6	24.99	24.7
	Polycarbonyl diacetyl $C_{16}H_{12}O_8N_8$	43.05	43.3	3.16	3.2	25.11	25.0

[1] Not enough purified sample was available to permit analysis of the other fractions listed in Table I.
[2] To conserve the small amount of sample, this analysis was not performed.
[3] Although analytical results of fraction 6 are reasonably compatible with values calculated for n-deca-2,4-dien-1-al-2,4-dinitrophenylhydrazone, its identity was not definitely established.

to little doubt. Gordon and coworkers *(6)* have shown that mixtures of these 2 compounds cannot be separated with the column chromatography method used here. Similar results were obtained when the authors attempted this separation with authentic samples. Hence separation of some pure n-butanal-2,4-dinitrophenylhydrazone (fraction 13) in this study from a mixture which apparently also contained the derivative of methyl-ethyl-ketone appears to have been a fortuitous occurrence. In any event on further column chromatography of fraction 14 neither pure n-butanal nor methyl-ethyl-ketone derivative could be obtained. However Lynn *et al. (7)* have shown that these 2 hydrazones may be readily separated by paper chromatography and when fraction 14 was so chromatographed, 2 well-separated spots, moving distances identical to those of authentic n-butanal and methyl-ethyl-ketone 2,4-dinitrophenylhydrazones were obtained. In addition the infrared spectrum of fraction 14 was compatible with the fact that it was a mixture of these 2 derivatives. These results, together with microscopic examination of properties of individual crystals obtained from fraction 14, indicate that it consists of a mixture of n-butanal and methyl-ethyl-ketone derivatives.

Although the remaining 2 monocarbonyl derivative fractions (Nos. 3 and 6) have not been definitely identified, they are believed to be derivatives of 2,4-dien-1-als because of their absorption spectra. This structural feature is indicated by their maximum ultraviolet absorption in chloroform solution at 389 mμ (1, 4, 5, 13). Their infrared spectra also agree with those of authentic n-hepta- and n-hexa-2,4-dien-1-al-2,4-dinitrophenylhydrazones with regard to two significant features distinguishing these 2,4-dien-1-al derivatives from authentic 2-en-1-al derivatives, namely, absence of a sharp distinct peak at 6.1 μ and the location of the out-of-plane C-H deformation frequency near 10.0 μ. Fractions 3 and 6 show melting points close to that reported for n-deca-2,4-dien-1-al-2,4-dinitrophenylhydrazone *(5, 9)*. Elemental analysis of fraction 6 (Table 2) is also compatible with its being this compound. The infrared spectra of fractions 3 and 6 differ only in the relative intensities

of 2 weak bands near 8.0 μ. The relative intensity of the C-H stretching bands near 3.4 μ with respect to the N-H stretching band at 3.05 μ for both fractions is about the same as for authentic n-deca-2-en-1-al-2,4-dinitrophenylhydrazone. This suggests a chain length of about 12 carbon atoms for these two 2,4-dienal derivatives. The above evidence suggests that fractions 3 and 6 are derivatives of 2,4-dien-1-als which are probably adjacent members of a homologous series or consist primarily of the same major component differing mainly in degree of purity. Conclusive identification of fractions 3 and 6 must await availability of authentic samples. In this connection, a general method of synthesizing 2,4-dien-1-als would be desirable. The authors had developed the method described in the experimental section before it was learned that recently Forss, Pont, and Stark *(5)* had developed a synthetic scheme which was identical except that they used manganese dioxide instead of chromic acid to oxidize the dienols to the corresponding aldehydes. They report that manganese dioxide converts the dienols to the aldehydes in yields of about 50%. Hence use of this oxidizing agent, instead of chromic acid, considerably improves the prospect of synthesizing the 2,4-dien-1-als in acceptable yields by this route.

DISCUSSION

To the authors' knowledge, the carbonyl compounds listed in Table I have not been previously identified among the volatile components of cooked chicken or of other cooked meats. In appraising the significance of these findings, some discussion of isolation procedures is necessary. In preliminary experiments the authors have isolated volatile carbonyl compounds, as the 2,4-dinitrophenylhydrazone derivatives, from chicken broth distillate and from steam evolved when chicken is simmered in water from 3 to 4 hours. Preliminary chromatography of these hydrazones indicated presence of a considerable number of carbonyl compounds. However, the yield of hydrazone obtained under these conditions was so low that these methods appeared to offer little hope of providing enough 2,4-dinitrophenylhydrazone to permit isolation and identification of minor components. It was then discovered that the yield could be increased by bubbling air through the system together with employment of longer cooking times. This "air entrainment" method was adopted for the present investigation. It is recognized that this isolation method involves a prolonged cooking time under conditions which favor oxidation. Volatile carbonyl compounds found under these conditions could be expected to include those which arise through oxidative processes in addition to or in greater amount than would be found in the corresponding fraction isolated from chicken cooked under normal conditions (cooking time 3 to 4 hours without passage of air through the cooking mixture). Hence carbonyl compounds identified here probably include both those which may constitute a part of normal flavor and those which may be associated with off-flavor.

Results obtained here raise several interrelated questions. One is determination of what contribution these carbonyl compounds make to poultry flavor. Another is the question of how the carbonyl fraction isolated from chicken cooked under normal conditions would compare to that obtained in this study.

Still another is the nature of the precursors which give rise to their formation. These questions cannot be answered until additional work is carried out. However, it is of interest to discuss them briefly in terms of available evidence.

One can postulate that fatty acids in the chicken fat are precursors of some of the aliphatic aldehydes and that the latter arise through oxidative cleavage at points of unsaturation in the fatty acid chain. This conjecture is based on the many reports in the literature concerning isolation of various carbonyl compounds from oxidized fats *(10)*. In this connection it should be mentioned that some of the carbonyl compounds isolated here, particularly the unsaturated aldehydes, have been associated with off flavors in oxidized fats *(10)* and in oxidized skim milk *(4, 5)*.

On the other hand, as mentioned above, the authors have isolated carbonyl compounds from the volatile components of chicken cooked under normal conditions. This means that carbonyl compounds are normally present to some extent among volatile components of cooked chicken and hence could constitute an important part of its characteristic aroma. It is important therefore to determine which carbonyl compounds are normally present and which may be associated with oxidized or rancid off flavors. It is expected that this will be the subject of a future investigation.

SUMMARY

Air was passed through a simmering mixture of chicken and water during a 20-hour period with passage of the exit gases through 2,4-dinitrophenylhydrazine solution. This procedure resulted in a yield of 7.8 g. of 2,4-dinitrophenylhydrazones from 31.4 kg. of chicken. Fractionation of the 2,4-dinitrophenylhydrazones resulted in the separation of 18 compounds. Conclusively identified were the derivatives of diacetyl, acetone, normal aliphatic saturated aldehydes containing 2, 3, 4, 5, 6, 8, and 9 carbon atoms, normal aliphatic 2-en-1-als containing 5, 6, 7, 10, and 11 carbon atoms, and n-hepta-2,4-dien-1-al. Evidence was also obtained indicating the presence of 2,4-dinitrophenylhydrazones of methyl-ethyl-ketone and of two unidentified 2,4-dien-1-als. These results provide new basic information about the identity of volatile components of cooked chicken and may be useful in determining the contribution that carbonyl compounds make to typical chicken flavor or off flavor.

Acknowledgment

The authors thank K. J. Palmer for X-ray diffraction data, H. J. Dutton for supplying an authentic sample of 2-pentenal-2,4-dinitrophenylhydrazone, L. M. White for ultimate analyses, and Edith Gong for obtaining the absorption spectra.

LITERATURE CITED

1. Braude, Ernest A., and Jones, E. R. H. Studies in light absorption. Part II. 2,4-Dinitrophenylhydrazones. *J. Chem. Soc.*, 498 (1945).
2. Bryant, W. D. Dynamic isomerism of acetaldehyde 2,4-dinitrophenylhydrazone. *J. Am. Chem. Soc.*, **60**, 2814 (1938).
3. Delaby, R., and Guillot-Allegre, S. Sur les aldehydes α,β-ethylénigues á chaine linéaire. *Bull. Soc. Chim. France*, **53**, 301 (1933).
4. Forss, D. A., Pont, E. G., and Stark, W. The volatile compounds associated with oxidized flavor in skim milk. *J. Dairy Research.* (Australia), **22**, 91 (1955).

5. Forss, D. A., Pont, E. G., and Stark, W. Further observations on the volatile compounds associated with oxidized flavor in skim milk. *J. Dairy Research* (Australia), **22**, 345 (1955).
6. Gordon, B. E., Wopat, Fred, Jr., Burnham, H. D., and Jones, L. C., Jr. Analysis of mixtures of aldehydes and ketones. *Anal. Chem.*, **23**, 1754 (1951).
7. Lynn, William S., Jr., Steele, Lois A., and Staple, Ezra. Separation of 2,4-dinitrophenylhydrazones of aldehydes and ketones by paper chromatography. *Anal. Chem.*, **28**, 132 (1956).
8. Malmberg, Earl W. A chromatographic study of carbonyl compounds present in a hydrocarbon-air flame. *J. Am. Chem. Soc.*, **76**, 980 (1954).
9. Martin, C. J., Schepartz, A. I., and Daubert, B. F. The preparation of 2-heptenal and 2-nonenal. *J. Am. Chem. Soc.*, **70**, 2601 (1948).
10. Morris, Steward G. Recent studies on the mechanism of fat oxidation in its relation to rancidity. *J. Agr. Food Chem.*, **2**, 126 (1954).
11. Newberg, Carl, and Strauss, Edward. Microdetermination of dicarbonyl compounds. On the coloring of nitro compounds in alkaline solutions. *Arch. Biochem.*, **7**, 211 (1945).
12. Pippen, E. L., and Eyring, E. J. Characterization of volatile nitrogen and volatile sulfur fractions of cooked chicken and their relation to flavor. *Food Technol.*, **11**, 53 (1957).
13. Roberts, J. D., and Green, C. Absorption spectra of some 2,4-dinitrophenylhydrazones. *J. Am. Chem. Soc.*, **68**, 214 (1946).
14. Staff, R. J., and Daubert, B. F. Flavor reversion in soybean oil. VI. Isolation and identification of reversion compounds in unhardened soybean oil. *J. Am. Oil Chem. Soc.*, **27**, 374 (1950).

EFFECT OF NON-ANTISCORBUTIC REDUCING SUBSTANCES UPON THE ASCORBIC ACID CONTENT OF BAKED POTATOES[a,b]

JANET WILSON McAFEE[c] AND JOHNNIE H. WATTS

The Carver Foundation, Tuskegee Institute, Alabama

(Manuscript received August 27, 1957)

A large volume of information concerning the effect of heat on the ascorbic acid content of vegetables has been accumulated. Of the various values reported, the effect of baking on the potato shows the greatest variation. Olliver *(6)* found losses ranging from 19-59% in the ascorbic acid content of baked potatoes. Hollinger *(2)* found that 76% of the ascorbic acid in the raw sweet potato is present in the baked potato. On the other hand, Scoular and Eakle *(11)* found in three varieties of baked sweet potatoes a 0.15-85% loss in ascorbic acid and in three other varieties, a 12-130% increase. Other investigators have found increases in ascorbic acid content of potatoes on baking *(3, 12)*.

This study was undertaken to determine the effect of baking on the true ascorbic acid content of potatoes. In the beginning phase of this work, it was found that baked potatoes, assayed with their peelings, showed an apparent gain in ascorbic acid when compared with raw potatoes. In view of this, a critical evaluation of the ascorbic acid content of potatoes baked to a series of internal temperatures was undertaken. Three different chemical methods were chosen for measuring the ascorbic acid content of potatoes. By use of these methods, the effect of non-antiscorbutic reducing substances upon the true ascorbic acid content of potatoes has been determined.

MATERIALS AND METHODS

Securing and handling of potatoes. Sweet potatoes, obtained from the experimental farm, Tuskegee Institute, were used in some of the experiments and white potatoes in others. White potatoes were not available at the school farm and were purchased, as needed, from local markets. Potatoes were cleaned, rinsed with glass-distilled water, wiped dry, weighed, and stored at 2° C. until the time of assay. At all times, analyses were made on both the raw and the baked potatoes.

Cooking. Baking was done in an electric table oven preheated to 450° F. and maintained at this temperature throughout the baking period. Internal temperature was determined with a Leeds and Northrup Precision Potentiometer, using a thermocouple consisting of 30-gauge iron and constantan wires. For experiments in which the potatoes were to be cooked to a series of 6 internal temperatures, 8 potatoes were matched for shape and weight. One was analyzed raw. The remaining 7 potatoes were weighed precisely, the thermocouple was inserted into one of them, and the 7 potatoes were put into the oven at the same time. Since the potatoes had been matched for shape and weight, it was assumed that the temperature of each potato was the same at all

[a] A portion of a thesis submitted by Janet Wilson McAfee to the Graduate Committee of Tuskegee Institute in partial fulfillment of the requirements for the degree of Master of Science.

[b] Supported by a Frederick Gardner Cottrell Grant from the Research Corporation.

[c] Present address: Department of Home Economics
University of California
Berkeley 4, California

times during the cooking period. As the e.m.f. corresponding to the desired temperature was reached, one of the potatoes which did not contain the thermocouple was withdrawn and assayed as the sample for that particular temperature.

For experiments in which the potato was to be cooked to doneness, namely 90° C., 2 potatoes similar in weight and shape were selected. One-half of one potato constituted the raw sample and the complementary half was weighed and wrapped in aluminum foil for baking and subsequent use as the cooked sample. One-half of the second potato was discarded and the thermocouple was inserted in the complementary half. When the e.m.f. corresponding to 90° C. was reached, the potato-half which had not been pierced by the thermocouple was removed and assayed.

Ascorbic acid assays. Extracts of plant tissues for vitamin C analyses are best obtained by thoroughly rupturing the cellular structure in the presence of acid. The acid protects the vitamin from rapid oxidation reactions which occur upon the liberation of active enzymes from the macerated cells *(1)*. For our work, C. P. grades of oxalic acid and metaphosphoric acid were used as extractants. A Waring blender was used to divide finely, and to mix uniformly the plant tissues in the acid extractant. The homogenates were filtered through fluted Whatman No. 12 filter paper and ascorbic acid determinations were made on the filtrates.

All solutions were made with glass-distilled water and potatoes were cut always with a stainless steel knife. The utmost care was taken to avoid metal contamination of the materials constituting the reaction mixtures.

The measurements of ascorbic acid were made with a Coleman Universal Spectrophotometer, Model 14. Three methods were chosen for the chemical assays: (a) the Loeffler and Ponting *(4)* adaptation of the 2,6-dichlorophenolindophenol method; (b) the indophenol xylene extraction method of Robinson and Stotz *(8)* with the formaldehyde modification for minimizing reductone interference; and (c) the Schaffert and Kingsley *(10)* modification of Roe and Oesterling's *(9)* 2,4-dinitrophenylhydrazine method.

DISCUSSION AND RESULTS

The ascorbic acid content of 6 replicate series of sweet potatoes heated

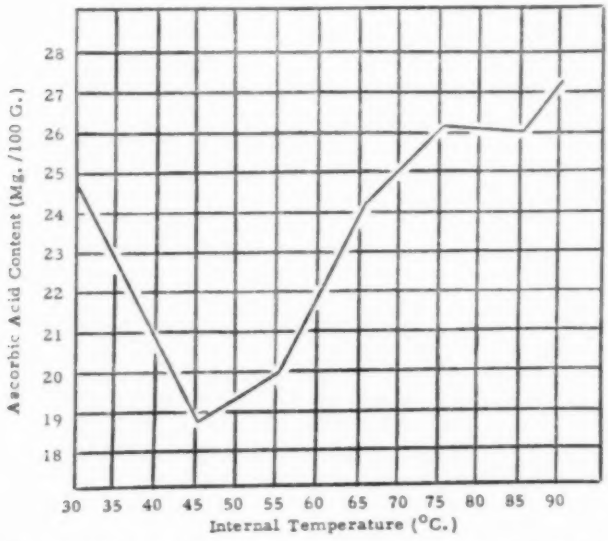


Figure 1. Ascorbic acid content of sweet potatoes baked to a series of internal temperatures.

to a series of internal temperatures, as determined by the procedure of Loeffler and Ponting, is reported in Figure 1. The average ascorbic acid content for raw sweet potatoes was 24.8 ± 9.1 mg./100 g. The average ascorbic acid content of sweet potatoes ranged from 18.8 ± 5.0 mg./100 g. for those baked to an internal temperature of 45° C. to 27.1 ± 6.6 mg./100 g. for those baked to an internal temperature of 90° C. Weights of potatoes were taken before baking; these values are for 100 g. potato, raw weight basis. Retention figures for the data in Figure 1 show that the ascorbic acid content of sweet potatoes baked to temperatures below 75° C. is less than that of raw sweet potatoes, whereas sweet potatoes baked to 75° C. and above contain more ascorbic acid than do the raw potatoes. The ascorbic acid content of the baked potato exceeded that of the raw potato at a temperature between 65° C. and 75° C. At all temperatures above 45° C., except at 85° C., there was a steady increase in ascorbic acid content. Above 45° C., it appears that an increase in internal temperature during baking is associated with an increase in ascorbic acid content. The average retention of ascorbic acid in potatoes cooked to doneness was 109%. This value confirms the data of Watts and Griswold *(12)*, Scoular and Eakle *(11)* and Leichsenring and co-workers *(3)* who reported retention values of 108%, 112-230%, and 109% in baked potatoes, respectively.

Roe and Oesterling *(9)* have suggested that higher values sometimes observed with the indophenol method for the determination of vitamin C in heated plant tissues are probably explained by the inactivation of ascorbase of the tissue, with resultant preservation of the reduced form of the vitamin. From the data in Figure 1, it would seem that ascorbase is active in the destruction of the vitamin at temperatures between 30° C. (room temperature) and 45° C. Ponting and Joslyn *(7)* report 43° C. as the temperature maximum for ascorbase activity and 6.0-6.8 as the range for pH optimum. In our experiments, conditions were favorable for the ascorbase oxidation of vitamin C at temperatures as high as 45° C. It does not seem feasible to assume that at temperatures above 45° C., the inactivation of enzymes could cause more of the vitamin to be present than at 45° C. Therefore, having determined ascorbic acid values at a series of temperatures between room temperature and the temperature of doneness, the increase in apparent ascorbic acid content is shown to be due to some factor other than enzyme inactivation. The increase in apparent ascorbic acid content of cooked potatoes is not shown when potatoes are cooked to doneness by moist heat, i.e., steaming, boiling, pressure-cooking *(12)*.

When total ascorbic acid—reduced plus dehydro—was determined in six replicates, it was found that the amount of total ascorbic acid in baked white potatoes exceeded the amount in raw white potatoes. For each replicate, the raw and the baked samples were obtained from respective halves of a single potato. The average total ascorbic acid content of the raw potatoes was 11.0 ± 3.3 mg./100 g. and the baked potatoes, 18.6 ± 7.3 mg./100 g., raw weight basis. The average retention was 173%.

Data obtained when potatoes were baked to an internal temperature of 90° C. and the ascorbic acid content was determined by the modified indophenol method of Robinson and Stotz are shown in Table 1. In this method, the oxidized indophenol was quantitatively extracted from one sample of

TABLE 1

Total reducing substances, reductones, and ascorbic acid in raw and baked white potatoes (mg./100 g., raw weight basis)

Replicates	Total reducing substances		Reductones		Reduced ascorbic acid	
	Raw	Baked	Raw	Baked	Raw	Baked
I	8.3	8.5	6.2	7.1	2.0	1.4
II	10.1	10.2	9.0	10.1	1.1	0.0
III	13.5	11.3	8.5	9.4	5.0	2.0
IV	16.9	13.2	14.7	13.1	2.2	0.1
V	25.1	26.3	21.5	23.1	3.5	3.2
VI	17.2	17.9	9.9	10.8	7.3	7.1
VII	13.1	15.9	10.9	15.3	2.1	0.6
Average	14.9 ± 5.6	14.8 ± 6.1	11.6 ± 5.1	12.7 ± 5.3	3.3 ± 2.2	2.1 ± 2.5

the potato filtrate with xylene. Formaldehyde was added to another sample of the same potato extract to react with reduced ascorbic acid, thereby removing it; the indophenol was then added and extracted, as before, to give a measure of the reductones present. Roe and Oesterling *(9)* have stated that the interference in chemical methods for the analysis of vitamin C, due to the interference of enediol-type compounds, is appreciable when carbohydrate-containing foods are heated in the presence of acid or alkali, and negligible in neutral or slightly acid solutions. Our results do not confirm this statement, since baking was done at the normal pH of the potato tissue which was 5.7. Data in Table 1 indicate that significant amounts of reducing substances, in addition to ascorbic acid, are present in both the raw and the baked potato filtrates. The extent of error in ascorbic acid determinations due to the presence of these non-antiscorbutic substances is shown: 23% of the reducing substances in raw potato filtrates and 14% of the reducing substances in baked potato filtrates is reduced ascorbic acid. The amount of dehydroascorbic acid, which also has antiscorbutic activity, was not determined in this procedure. The use of methods that do not alleviate the effect of reductones tends to indicate that all reducing substances present are ascorbic acid. The magnitude of the error in using such methods is large indeed. Total reducing substances, as determined by the Robinson and Stotz method, are approximately the same in raw and baked potato filtrates, the retention being 99%.

Whereas more reductone-type compounds than reduced ascorbic acid are present in raw potato filtrates, on baking there is a concomitant increase in reductone-type reducing substances and a decrease in reduced ascorbic acid—the reductone-type reducing substances show a 10% increase on baking, while reduced ascorbic acid shows a 38% decrease.

Attempts were made to remove and isolate from both the raw and the baked potato filtrates the non-antiscorbutic factors. The filtrates were concentrated and paper chromatograms were prepared. Chromatographic separations were unsuccessful, mainly because of the high concentrations of substances other than those of interest at the time. Further attempts at separation and identification of the non-antiscorbutic reducing substances will be pursued in the future.

SUMMARY

On measuring the ascorbic acid content of potatoes baked to a series of internal temperatures between 45° C. and 90° C., it was observed that there is a decrease in ascorbic acid content at the beginning of the baking period and thereafter, an increase. The retention of reduced ascorbic acid in baked sweet potatoes, as determined by the indophenol method, was 109%. The retention of total ascorbic acid—reduced + dehydro—in baked white potatoes was 173%. The retention of total reducing substances in baked white potatoes was 99%, as determined by the indophenol-xylene extraction method; however, when the formaldehyde modification of this same method was used, the retention of reductone-type compound was 110% and of reduced ascorbic acid, 62%.

Dehydration at high temperatures, which occurs during baking, might be the causative factor in the formation of these non-antiscorbutic reducing substances. Data obtained by three different methods emphasize the importance of having a suitable method for making ascorbic acid analyses.

LITERATURE CITED

1. Davis, W. B. Extraction of ascorbic acid from plant tissues. *Ind. Eng. Chem., Anal. Ed.*, **34**, 217 (1942).
2. Hollinger, M. E. Ascorbic acid value of the sweet potato as affected by variety, storage, and cooking. *Food Research*, **9**, 76 (1944).
3. Leichsenring, J. M., H. L. Pilcher, and L. M. Norris. II. Effect of baking and of pressure-cooking on the ascorbic, dehydroascorbic, and diketogulonic acid contents of potatoes. *Food Research*, **22**, 44 (1957).
4. Loeffler, H. J., and J. D. Ponting. Ascorbic acid. Rapid determination in fresh, frozen, or dehydrated fruits and vegetables. *Ind. Eng. Chem., Anal. Ed.*, **14**, 846 (1942).
5. McHenry, E. W., and M. Graham. Observations on the estimation of ascorbic acid by titration. *Biochem. J.*, **29**, 2013 (1935).
6. Olliver, M. Effect of cooking on the nutritive value of vegetables. *Chemistry and Industry*, 586 (1941).
7. Ponting, J. D., and M. A. Joslyn. Ascorbic acid oxidation and browning in apple tissue extracts. *Arch. Biochem.*, **19**, 47 (1948).
8. Robinson, W. B., and E. Stotz. The indophenol-xylene extraction method for ascorbic acid and modifications for interfering substances. *J. Biol. Chem.*, **160**, 217 (1945).
9. Roe, J. H., and M. J. Oesterling. The determination of dehydroascorbic acid and ascorbic acid in plant tissues by the 2,4-dinitrophenylhydrazine method. *J. Biol. Chem.*, **152**, 511 (1944).
10. Schaffert, R. R., and G. R. Kingsley. A rapid, simple method for the determination of reduced, dehydro-, and total ascorbic acid in biological material. *J. Biol. Chem.*, **212**, 59 (1955).
11. Scoular, F. I., and D. H. Eakle. Loss of ascorbic acid during cooking of stored sweet potatoes. *Food Research*, **8**, 156 (1943).
12. Watts, J. H., and R. M. Griswold. Enzyme inactivation. Relation of rates of inactivation of peroxidase, catecholase, and ascorbase to oxidation of ascorbic acid in potatoes and parsnips. *J. Agr. Food Chem.*, **1**, 569 (1953).

OPTIMUM CONDITIONS OF HYDROLYSIS FOR MICROBIOLOGICAL ASSAY OF CYSTINE IN FOOD PROTEINS[a]

S. C. BALASUBRAMANIAN[b] AND M. RAMACHANDRAN[b]

Food Technology Laboratory, Department of Biochemistry, Indian Institute of Science, Bangalore, India

(Manuscript received March 9, 1957)

Cystine, though not classified as one of the essential amino acids, has been shown to spare the human needs for methionine to a considerable extent *(21)*. It is well-known that tropical diets predominantly composed of vegetable foods, especially cereals, are deficient in methionine *(8)* and hence the need arises to investigate the cystine content of such foods. While undertaking such an analysis by the microbiological procedure, certain observations regarding the conditions of hydrolysis and the method to be adopted were made by the authors. These are presented below.

EXPERIMENTAL

Previous investigations. Earlier work by the authors on the microbiological assay of amino acids in Indian foods had shown that the method of Barton-Wright *(4)* was readily applicable with some minor modifications. However, for cystine, this method proved unsuccessful, high titres being obtained in the blank tubes. Several methods described in the literature *(5, 7, 12, 13, 17, 19, 20, 22)* were tried but did not yield satisfactory assays. Heathcote *(11)* reported that the use of synthetic media in assays of cystine gave erratic values. A medium utilizing peroxide-treated peptone as source of amino acids and an average value of several independent determinations was suggested to be the best. The present writers also found that peroxide-treated peptone medium was the best and since the standard curves were not always superimposable, at least three or even more independent determinations were necessary to arrive at a satisfactory value. Recently Horn and Blum *(14)* have used a hydrolysate of lanthionised casein as source of amino acids.

The basal medium. 'Difco' Bacto-peptone was treated with hydrogen peroxide according to the procedure of Lyman *et al. (16)*. This was used as the source of amino acids in the place of oxidized casein (in equal amounts) in medium V of Sauberlich and Baumann *(22)*. Satisfactory assays with reproducible standard curves resulted.

Assay details. *Leuconostoc mesenteroides* strain P-60[c] was used as the test organism and the assay procedures were similar to those described earlier by Balasubramanian *et al. (3)*. The total volume per tube was 5 ml. made up of 2.5 ml. of basal medium and 2.5 ml. of standard solution or hydrolysate plus water. The range of the standard curve was 0-12 μg. l-cystine, the usable portion thereof being 0-5 μg. The solution of cystine used for the standard curve contained 5 μg/ml. of l-cystine in acidulated water. A typical standard curve appears in Figure 1.

Hydrolysis. As with the other assays, the hydrolysates of rice and wheat were prepared by autoclaving 1 g. of the finely ground flour for 6 hrs. with 25 ml. of 2.5N HCl

---

[a] Part of a study made by one of us (S.C.B.) in fulfillment of the requirements for the Ph.D. degree, the University of Bombay, India.

[b] *Present address:* Nutrition Research Laboratories, Coonoor, India.

[c] No. 2177 obtained from National Collection of Type Cultures (India), National Chemical Laboratory, Poona.

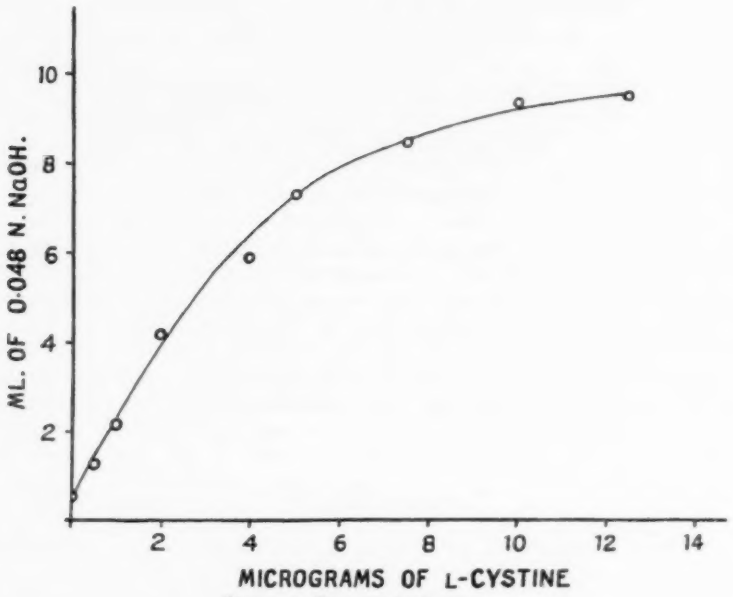


Figure 1. The standard curve.

and the assays carried out. Very low values in comparison with literature values were obtained.

This prompted an investigation of the conditions for hydrolysis of food materials to obtain the best results. Alkali destroys cystine; hence, it is unsuitable as an hydrolyzing agent. Hydrolysis by acid in the presence of large amounts of carbohydrates as occurs in complex materials like foodstuffs also leads to loss of cystine *(15)*. Miller *et al. (18)* found that autoclaving of wheat with 4N HCl for 1 hr. gave maximum values of cystine. It was felt by the present writers that maximum values need not necessarily reflect maximum liberation of cystine and subsequent destruction inasmuch as peptides of amino acids have been reported to give higher response than the corresponding equivalent quantity of amino acid in microbiological methods *(9)*. Consequently, maximum values might represent completely liberated cystine with or without admixed peptides of cystine. A solution to this problem would have been to adopt a method capable of estimating free cystine in the presence of its peptides. The highly specific Sullivan's method would not be sensitive enough to detect cystine in food protein hydrolysates. Recovery experiments with added cystine would be fallacious since peptide cystine undergoes quicker destruction by acid in the presence of carbohydrates *(2)* than free cystine.

The approach. Hence, it was thought that a simultaneous examination of cystine and amino nitrogen liberated after hydrolysis of the cereal samples with 2.5N HCl for stated periods would reveal the optimum hydrolysis conditions for the maximum release of cystine.

Preparation of samples. One gram samples each of rice and wheat were hydrolyzed for 1, 2, 3, and 6 hrs. at 15 lbs. pressure and the hydrolysates were prepared for assay as described in an earlier communication *(3)*. A mixture of 900 mg. of pure starch, 2 ml. of peroxide-treated peptone used for the basal medium (corresponding to 100 mg. peptone), 1.5 mg. each of dl-methionine and dl-tyrosine, 2.0 mg. of dl-tryptophane and 2.0 mg. of l-cystine was similarly autoclaved with HCl for the different periods to simulate a system of free cystine in the presence of carbohydrates and other amino acids as is likely to occur during the hydrolysis of a cereal. The degree of loss of cystine was determined at the

end of different periods. In the hydrolysates of rice and wheat the amino nitrogen was determined by the copper method *(1)*. Three independent trials were carried out and the average figures obtained in the three assays were used in the calculations.

RESULTS AND DISCUSSION

Cystine in rice and wheat. The data on cystine and amino nitrogen content are presented in Table 1.

TABLE 1

Effect of time of hydrolysis at 120°C on the release of cystine and amino nitrogen.

Material	Time of hydrolysis			
	1 hr.	2 hrs.	3 hrs.	6 hrs.
	Cystine content (mg. per cent).			
Rice	108	183	140	37
Wheat	171	232	202	76
Starch-peptone-amino acids-cystine system (see text) (2 mg. cystine)	2.0 mg.	1.9 mg.	1.9 mg.	1.45 mg.
	Amino Nitrogen (per cent of total nitrogen).			
Rice	44.6	49.1	52.1	53.6
Wheat	46.3	53.6	55.8	55.8

Assuming that the amino nitrogen released after 6 hrs. was 100% in the case of the rice and wheat samples, the percentage release of amino nitrogen at the end of 1, 2 and 3 hrs. was calculated and plotted against time. The amount of cystine liberated at the end of various periods was also plotted in the same graph (Figs. 2 and 3). Both the curves intersect at more than one point. It

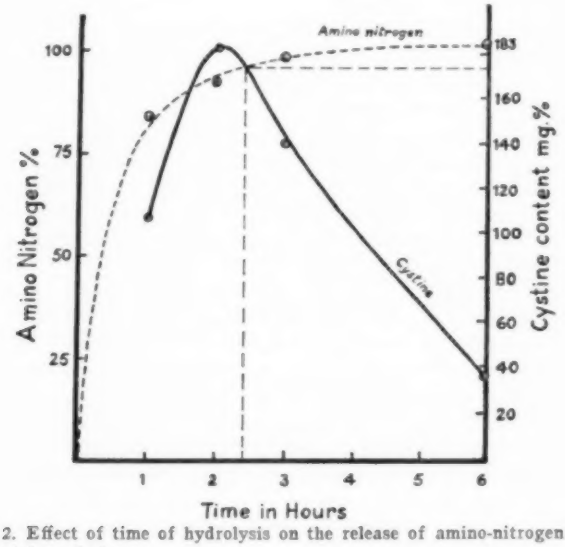


Figure 2. Effect of time of hydrolysis on the release of amino-nitrogen and cystine from the proteins of rice.

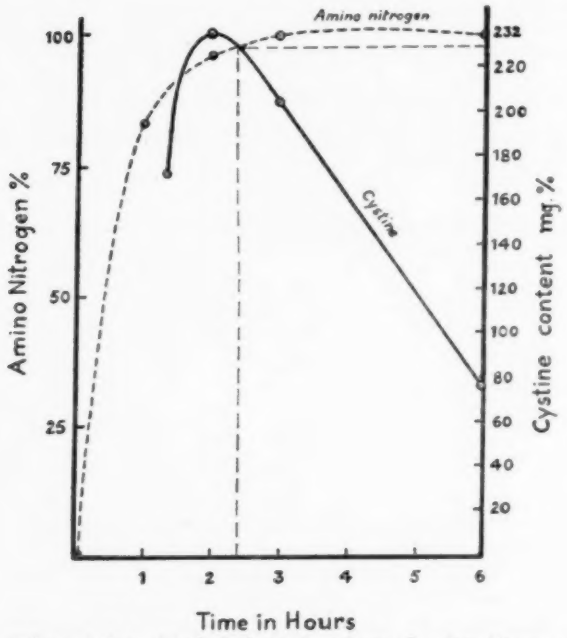


Figure 3. Effect of time of hydrolysis on the release of amino-nitrogen and cystine from the proteins of wheat.

was considered that at a point nearer the maximum degree of hydrolysis peptide-bound cystine is likely to be present in the least amounts and as such, this point of intersection may represent the best value for free cystine in the material concerned. At the earlier points of intersection, since the hydrolysis has not reached its peak as revealed by the amino nitrogen data, the cystine content may be made up of the free form with or without the peptide-bound form and thus may not represent the true value. Such an approach presupposes a belief that the release of amino nitrogen will be concomitant with the release of cystine, and thus enable it to be estimated at a time when maximum liberation and minimum destruction have occurred.

Optimum conditions. The experiments with a starch-peptone-amino acids-cystine system indicated that destruction of free cystine occurs only after hydrolysis with acid for 2 hrs. The results obtained with rice and wheat showed that hydrolysis for a period between 2¼ and 2½ hrs. with 2.5N HCl might represent the optimum conditions for hydrolyzing cereals to get the best representative values for cystine. For reasons not accessible to the present writers, Hankes *et al. (10)* also adopted hydrolysis for 3 hrs. with 2N HCl for estimating cystine. These conditions are also akin to what have been observed in the present study.

The cystine content of rice and wheat as obtained from the curves (Figs. 2 and 3) is given in Table 2. The literature values appear to be rather low

TABLE 2

Cystine content of rice and wheat.

Cereal	Cystine content		Literature values per cent[3]
	In foodstuff mg. per cent[1]	In protein per cent[2]	
Rice	174	1.8	1.1 - 1.5 (1.3)
Wheat	226	2.2	1.0 - 2.6 (1.8)

[1] Values obtained from Figures 2 and 3 and uncorrected for moisture and ash.
[2] After calculation to 16% nitrogen basis.
[3] Literature values are from Block and Bolling *(6)* on 16% nitrogen basis. The most representative value is given in parentheses.

compared to the present values perhaps due to some possible destruction of cystine during hydrolysis not having been accounted for by the earlier workers.

Studies with fibrin and egg albumin. Experiments were also carried out on the effect of time of hydrolysis on the release of cystine from two pure proteins, fibrin and egg albumin. The relevant data appear in Table 3. The maximum amount of microbiologically estimable cystine was obtained by hydrolysis for 2 hrs. with 2.5N HCl. However, the values obtained after

TABLE 3

Cystine content of fibrin and egg albumin.

Protein	Cystine (per cent) Time of hydrolysis at 120°C.				Literature values per cent[1]
	1 hr.	2 hrs.	3 hrs.	6 hrs.	
Fibrin[2]	2.10	2.55	2.20	1.75	1.2 - 3.1 (1.7)
Egg albumin[2]	2.85	3.10	2.00	1.65	0.9 - 3.0 (2.5)

[1] Literature values are from Block and Bolling *(6)* for the proteins as such. In recalculating these values, the total nitrogen of fibrin and egg albumin were assumed to be 17.7 and 15.4% respectively. The most representative value is given within parentheses.
[2] Fibrin and egg albumin were commercial samples manufactured by British Drug Houses Ltd., London.

hydrolysis of fibrin for approximately 6 hrs. and egg albumin between 2 and 3 hrs. appear to compare well with literature values. The true value can perhaps be best found by carrying out a parallel study of amino nitrogen release also as was done with rice and wheat.

SUMMARY

Failure was encountered using several methods reported in the literature for the microbiological assay of cystine. A medium prepared from hydrogen peroxide treated peptone was found to be the best for the assay with *L. mesenteroides* strain P-60.

The usual 6 hr. hydrolysis of cereals resulted in lowered values. A simultaneous study of the amino nitrogen and cystine released from rice and wheat

by hydrolysis with 2.5N HCl for various periods indicated 2½ hours as the optimum period for hydrolysis.

Experiments with two pure proteins, fibrin and egg albumin also pointed to the need for a parallel study of amino nitrogen release to arrive at the true cystine value.

Acknowledgment

The authors wish to thank Dr. K. V. Giri, Professor of Biochemistry, Indian Institute of Science, Bangalore for kind encouragement in the progress of the work, Dr. M. Sirsi for help in maintenance of cultures and the Indian Council of Medical Research for generous grants-in-aid in support of this investigation.

LITERATURE CITED

1. Albanese, A. A., and Irby, V. Determination of urinary amino nitrogen by the copper method. *J. Biol. Chem.*, **153**, 583 (1944).
2. Bailey, K. The sulphur distribution of proteins. *Biochem. J.*, **31**, 1396 (1937).
3. Balasubramanian, S. C., Ramachandran, M., Viswanatha, T., and De, S. S. Amino-acid composition of Indian foodstuffs. I. Tryptophane, leucine, isoleucine and valine content of some cereals. *Indian J. Med. Research* **40**, 73 (1952). II. Lysine, methionine, phenylalanine and histidine content of some cereals. *Indian J. Med. Research*, **40**, 219 (1952).
4. Barton-Wright, E. C. The microbiological assay of amino acids. I. The assay of tryptophane, leucine, isoleucine, valine, cystine, methionine, lysine, phenylalanine, histidine, arginine and threonine. *Analyst*, **71**, 267 (1946).
5. Barton-Wright, E. C., and Curtis, N. S. Modified method for the microbiological assay of tryptophane, methionine, cystine and tyrosine. *Analyst*, **73**, 330 (1948).
6. Block, R. J., and Bolling, D. The Amino Acid Composition of Proteins and Foods. 2nd ed., 1951. Charles C. Thomas, Springfield, Illinois.
7. Camien, M. N., and Dunn, M. S. An improved microbiological method for the determination of cystine in human urine with *Leuconostoc mesenteroides* P-60. *J. Biol. Chem.*, **183**, 561 (1950).
8. Carpenter, K. J. The relative nutritional values of animal and vegetable proteins for animals. *British J. Nutrition*, **5**, 243 (1951).
9. Dunn, M. S., and McClure, L. E. The response of *Lactobacillus casei* to partial hydrolysates of protein. *J. Biol. Chem.*, **184**, 223 (1950).
10. Hankes, L. V., Riesen, W. H., Henderson, L. M., and Elvehjem, C. A. Liberation of amino acids from raw and heated casein by acid and enzyme hydrolysis. *J. Biol. Chem.*, **176**, 467 (1948).
11. Heathcote, J. G. The toxicity of small concentrations of cystine to acid producing bacteria. *J. Gen. Microbiol.*, **3**, 392 (1949).
12. Heller, G. L., and Kirch, E. R. The microbiological determination of proline, cystine and isoleucine. *J. Am. Pharm. Assoc., Sci. Ed.*, **36**, 345 (1947).
13. Hift, H., and Wallace, G. I. A study on the synthesis of cystine by some lactic acid bacteria. *J. Biol. Chem.*, **177**, 927 (1949).
14. Horn, M. J., and Blum, A. E. A microbiological method for determination of cystine in foods. *Cereal Chem.*, **33**, 18 (1956).
15. Lugg, J. W. H. Some sources of error in the estimation of cysteine and cystine in complex materials when acid hydrolysis is employed. *Biochem. J.*, **27**, 1022 (1933).
16. Lyman, C. M., Moseley, O., Wood, S., and Hale, F. Note on the use of hydrogen-peroxide treated peptone in media for the microbiological determination of amino acids. *Arch. Biochem.*, **10**, 427 (1946).
17. Mahadevan, V. Microbiological determination of cystine in foods and proteins. *J. Sci. and Ind. Res.*, (India) **9B**, 143 (1950).
18. Miller, B. S., Seiffe, J. Y., Shellenberger, J. A., and Miller, G. D. Amino acid content of various wheat varieties. I. Cystine, lysine, methionine and glutamic acid. *Cereal Chem.*, **27**, 96 (1950).

19. RAM MOHAN, R., THADHANI, K. C., GOVINDARAJAN, V. S., AND SREENIVASAYA, M. Protein hydrolysates for the microbiological assay of amino acids. *Current Sci.*, **18**, 427 (1949).
20. RIESEN, W. H., SPENGLER, H. H., ROBBLEE, A. R., HANKES, L. V., AND ELVEHJEM, C. A. Cystine and related compounds in the nutrition of lactic acid bacteria. *J. Biol. Chem.*, **171**, 731 (1947).
21. ROSE, W. C., AND WIXON, R. L. The amino acid requirements of man. XIII. The sparing effect of cystine on the methionine requirement. *J. Biol. Chem.*, **216**, 763 (1955).
22. SAUBERLICH, H. E., AND BAUMANN, C. A. The effect of dietary protein upon amino acid excretion by rats and mice. *J. Biol. Chem.*, **166**, 417 (1946).

NOTES AND LETTERS

EFFECT OF METABOLITES ON THE VIABILITY OF HEAT-TREATED PSEUDOMONAS FLUORESCENS[a]

Recent investigations *(3, 4)* indicate that the characteristics of the plating medium and the time and temperature of plate incubation may affect the enumeration of heat-treated pseudomonads. Heinmets *et al. (5)*, however, reported that *Escherichia coli* B/r inactivated by heat, chlorine or ethanol could be reactivated when incubated with certain Krebs cycle metabolites. Garvie *(2)* reported multiplication of chlorine-treated cells of *E. coli* in buffer solutions plus metabolite and considered the "reactivation" process the result of the multiplication of a few survivors. In this study the effect of metabolites on heat-treated *P. fluorescens* (strains 2 and 3) was investigated.

The cultures were grown in a broth containing glucose 5 g., $NH_4H_2PO_4$ 1 g. KH_2PO_4 1 g., water 1 l., pH 7.0. The growth which developed after 24 hours at 25° C. was collected by centrifugation, washed twice with ice-cold phosphate buffer (0.0003M, pH 7.0) and re-suspended in buffer. In the heating trials one ml. of this suspension was added to 99 ml. of hot phosphate buffer (pH 7.0) adjusted to the desired temperature in a constant temperature waterbath. The suspension was agitated continuously by a stirrer. Samples were withdrawn into sterile screw-cap test tubes submerged in ice water.

Aliquots of the samples before and after heating were plated on Tryptone Glucose Yeast (TGY) and "Synthetic" agar (SY) which contained the same ingredients as the broth with 15 g. of agar per 1. Aliquots of the non-heated and heated cultures were added (1:1 ratio) to phosphate buffer (pH 7.0) and buffer plus various additives (0.2%). The mixtures were incubated at 25° C. for 3 days. The various materials were sterilized by filtration through Seitz filters. The heat treatment was considered lethal when one ml. of the culture did not show survivors on TGY agar and did not show growth in nutrient broth after incubation at 25° C. for 5 days. If no visible growth was present in nutrient broth one ml. of the broth was plated on TGY agar. Incubation of a few survivors (Figure 1, 55° C., 5 min., and 60° C., 1.5 min.) with a mixture of 11 metabolites considerably increased the number of viable cells. No viable cells could be detected in suspensions which before incubation with metabolites did not show survivors on TGY agar (60° C., 2 min.). The pattern of response of non-heated cultures to incubation with buffer and buffer plus metabolites was very similar to that obtained with cultures heated a 55° C.

A considerable increase in viable cells was observed when heated suspensions were incubated with isocitric acid, sodium citrate or a mixture of 11 metabolites (Table 1). Growth, however, was observed only when survivors were found on TGY agar before incubation with metabolites. It is unlikely that these compounds merely protect the cells from the destructive action of common diluting fluids, in view of the marked increase in the number of

[a] Journal Paper No. 2716 of the Texas Agricultural Experiment Station, College Station.

TABLE 1

Effect of incubating heated suspensions of *P. fluorescens* 2 with metabolites on the number of viable cells.

Heated sample incubated with	No. of viable cells per ml. after incubation					
	1.5 min. at 60° C.		2 min. at 60° C.		Control	
	TGY agar	Synthetic agar	TGY agar	Synthetic agar	TGY agar	Synthetic agar
Buffer	<1	<1	<1	<1	<1	<1
***Cis*-Aconitic acid**	<1	<1	<1	<1	<1	<1
Isocitric acid	160×10^1	110×10^1	<1	<1	32×10^8	49×10^8
α-Ketoglutaric acid	<1	<1	<1	<1	<1	<1
Lactic acid	<1	<1	<1	<1	<1	<1
Malic acid	<1	<1	<1	<1	<1	<1
Oxalacetic acid	<1	<1	<1	<1	<1	<1
Sodium acetate	<1	<1	<1	<1	<1	<1
Sodium citrate	42×10^8	35×10^8	<1	<1	223×10^8	195×10^8
Sodium fumarate	<1	<1	<1	<1	<1	<1
Sodium pyruvate	<1	<1	<1	<1	<1	<1
Succinic acid	<1	<1	<1	<1	<1	<1
Mixture of 11 metabolites	51×10^8	42×10^8	<1	<1	181×10^8	159×10^8

No. of viable cells per ml. on	TGY agar	Synthetic agar
Before heating	39×10^8	42×10^8
After heating (1.5 min.)	13	<1
After heating (2 min.)	<1	<1
Control (unheated)	53	32

viable cells after incubation with certain metabolites. The possibility exists that these compounds provide some form of readily available energy.

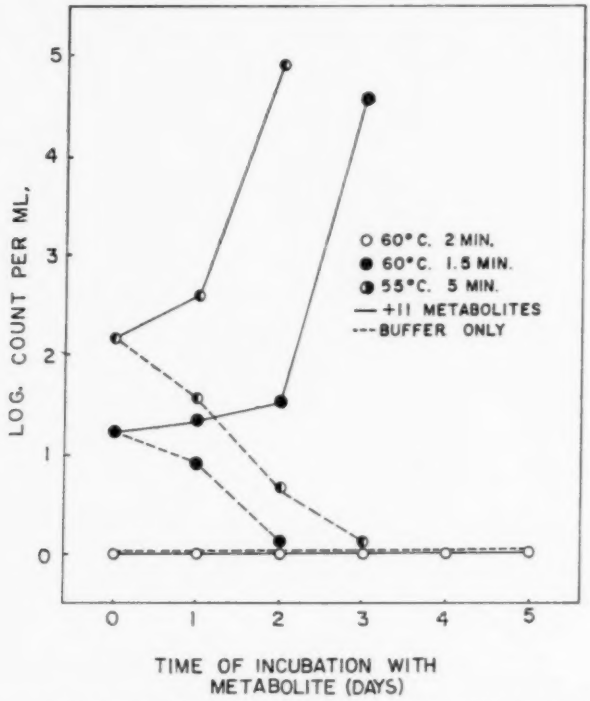


Figure 1. Effect of incubating heat-treated *P. fluorescens* 2 with buffer and metabolites[1] on the number of viable cells[2].

[1] See Table 1.
[2] Counts were made on tryptone glucose yeast agar.

Subsequent experiments showed a considerable increase in viable cells when a few heat-treated survivors were incubated with beef extract, casamino acids, nutrient broth, synthetic broth or yeast extract. At no time were viable cells detected when the heated suspensions before incubation with nutrients or metabolites did not show survivors on TGY agar. The data presented are representative of those obtained in five independent trials. Recently, Chambers *et al. (1)* also were unable to obtain reactivation of "killed" cells of *E. coli* by incubation with various metabolites.

October 25, 1957

C. D. HEATHER
C. VANDERZANT
Department of Dairy Science
Texas A and M College System
College Station, Texas

LITERATURE CITED

1. CHAMBERS, C. W., TABAK, H. H., AND KABLER, P. W. Effect of Krebs cycle metabolites on the viability of *Escherichia coli* treated with heat and chlorine. *J. Bacteriol.*, **73**, 77 (1957).
2. GARVIE, E. I. Growth of *Escherichia coli* in buffer substrate and distilled water. *J. Bacteriol.*, **69**, 393 (1955).
3. HEATHER, C. D., AND VANDERZANT, C. The effect of the plating medium on the survival of heat-treated cells of *Pseudomonas fluorescens*. *Food Research*, **22**, 164 (1957).
4. HEATHER, C. D., AND VANDERZANT, C. The effects of temperature and time of plate incubation and the pH of the plating medium on the enumeration of heat-treated psychrophilic bacteria. *J. Dairy Sci.* (In Press).
5. HEINMETS, F., TAYLOR, W. W., AND LEHMAN, J. J. The use of metabolites in the restoration of the viability of heat and chemically inactivated *Escherichia coli*. *J.* Bacteriol., **67**, 5 (1954.)

THE ROLE OF METHIONAL AS A FLAVOR COMPOUND[a]

The importance of methional (3-methylthiopropanal) as a flavor compound in certain foods has been suggested in recent years. Patton *(6)* reported that the compound exhibited a broth-like flavor at the proper concentration and suggested that it may have general significance in the flavor of a variety of foods. Recently, Keeney and Day *(2)* suggested that methional may play a prominent role in cheese flavor. Consequently, the article by Witting and Batzer *(9)*, suggesting that methional sulfoxide (3-methylsulfinylpropanal) instead of methional is the flavor compound and their claim that methional is odorless, pointed to the necessity for re-evaluation of previous work and the accumulation of additional data in order to clarify the problem. The results of our investigation are contrary to the proposal of Witting and Batzer *(9)* and verify reports *(2, 5, 6)* that methional is a potent organoleptic compound.

Fresh methional was synthesized from acrolein and methyl mercaptan by the procedure of Pierson *et al. (8)*. The crude product was purified by distillation through a fractionating column packed with glass helices at 40° C. and 8 mm pressure and was collected into a receiving flask cooled by an ice bath. A portion of the methional was placed into vials, sealed and refrigerated until used for infrared and mass spectrometric analyses; the remainder was used for immediate studies which included odor and flavor observations, determinations of refractive index and density, and preparation of the 2,4-dinitrophenylhydrazone (2,4-DNPH). These studies were conducted on both the fresh sample and a sample after exposure to air for two hours.

The cold receiving flask was examined (4 observers) immediately after breaking the vacuum and a strong odor, previously associated with methional from the Strecker degradation of methionine, was noted. The odor of the flask did not change after prolonged exposure to air. The refractive index $n\frac{20}{D}$ 1.4800, $n\frac{30}{D}$ 1.4770 was constant for both the fresh and the air exposed material. The density was $d\frac{20}{4}$ 1.053, $d\frac{30}{4}$ 1.049. The molecular refractivity of the air exposed methional was 28.6 at 20° C., while the theoretical for methional is 28.66 *(4)*.

A portion of the air exposed methional was reacted with a 1N H_2SO_4 solution of 2,4-dinitrophenylhydrazine and the crude product was recrystallized from aqueous methanol solutions to a constant melting point of 121-121.5° C. (corrected). Elemental analysis of the 2,4-DNPH gave the following percentages: C = 42.46, H = 4.16, N = 19.69, S = 11.00, O = 22.69 (calc.). Theoretical values for the 2,4-DNPH of methional ($C_{10}H_{12}O_4N_4S$) are: C = 42.25, H = 4.25, N = 19.71, S = 11.28, O = 22.51, and for the 2,4-DNPH of methional sulfoxide ($C_{10}H_{12}O_5N_4S$) are: C = 39.99, H = 4.04, N = 18.66, S = 10.68, O = 26.64.

Equal quantities of the purified 2,4-DNPH were used to regenerate the

---

[a] Scientific Article No. *A. 648*, Contribution No. *2837*, of the Maryland Agricultural Experiment Station, Dairy Department.

free methional by the levulinic acid method of Keeney *(3)* under atmospheres of nitrogen and oxygen. Each gas was bubbled into a regeneration tube and the liberated methional was trapped in a U tube cooled by an ethanol-dry ice bath. In both cases the cold traps possessed a strong odor which we attributed to methional.

Infrared spectra of fresh methional and a sample which had been flushed with oxygen for 45 minutes were completely superimposable. Consequently, it was believed that if minute changes were occurring in methional the mass spectrometer would be best suited for their detection due to its high sensitivity. Any oxidation products of methional should be readily detected since the parent peaks are at m/e 104 for methional, 120 for methional sulfoxide or 3-methylthiopropionic acid, 136 for methional sulfone (3-methylsulfonylpropanal) or the sulfoxide of 3-methylthiopropionic acid (3-methylsulfinylpropionic acid), and 152 for the sulfone of the acid (3-methylsulfonylpropionic acid). Furthermore, the percentages of impurities are easily determined. A consolidated Model 21-103 Mass Spectrometer was used to obtain spectra of a fresh methional sample and an oxygen flushed sample. Both spectra exhibited base peaks at m/e 48 and major parent peaks at 104. Some impurities were noted in the range of m/e 112 to 140 but neither spectrum gave evidence for methional sulfoxide (m/e 120). The largest peak of the impurities was m/e 136 (.007% for the fresh sample and .029% for the oxygenated sample) which is indicative of methional sulfone or the sulfoxide of 3-methylthiopropionic acid.

The insignificance of the observed impurities in the methional odor was demonstrated by application of the flavor threshold procedure described by Patton and Josephson *(7)*. The flavor threshold value of methional was determined using fresh pasteurized homogenized milk as the diluent. A value of 16 p.p.b. was found as the average threshold at the 50% level. Similar values were observed with water as the diluent. If the impurity rather than methional gave the flavor, the threshold would be in the range of 1 to 5 p. p. tr. Such a low concentration is beyond the detectable range for any compound ever reported. It is noteworthy, that during this determination of threshold some of the observers characterized the samples containing low levels of methional (10-40 p.p.b.) as having sunlight-milk flavor, while the samples containing high levels (1-2 p.p.m.) were characterized as cheesy.

For a comparative study, the authors are grateful to Witting and Batzer for a sample of the methional used in their investigation *(9)*. Two mass spectra were obtained, one before and one after exposure to air for 5 days. Both spectra were comparable to ours with respect to base and parent peaks and the lack of evidence for methional sulfoxide. The Witting sample showed more impurity in the lower m/e region and the largest peak in the higher m/e region was 136 which is indicative of the sulfone or sulfoxide-acid. It should also be pointed out that, in the opinion of the authors, the Witting sample exhibited an abundance of odor when the vial was first opened.

The evidence accumulated in this investigation is in direct disagreement with the findings of Witting and Batzer *(9)*. The strong odor of the freshly distilled methional and of that which was regenerated from its 2,4-DNPH under nitrogen, indicate that oxidative conditions are not required for odor.

The fact that bubbling oxygen through a sample for 45 minutes produced a change in possible oxidation products (none of which was methional sulfoxide) of only .022% suggests that the thio ether linkage is rather stable to oxidative conditions. This fact was previously demonstrated by Witting and Batzer *(9)* in the preparation of the methyl ester of 3-methylthiopropionic acid when they employed 30% hydrogen peroxide to oxidize methional to the acid prior to esterification. If the thio ether linkage was oxidized as readily as proposed, it would be difficult to obtain an appreciable yield of 3-methylthiopropionic acid.

Information in the literature *(1)*, as well as general experience, support the fact that as the sulfur in a molecule is oxidized, the odor diminishes and that sulfoxides are odorless. Sulfides, in general, are more stable than sulfoxides to oxidation. It appears that methional is no exception since the evidence presented proves it to be an odorous compound. Furthermore, the presence of methional sulfoxide in methional preparations could not be demonstrated under any of the conditions employed.

October 25, 1957

E. A. DAY
MARK KEENEY
Dairy Department, University of Maryland
College Park, Maryland
WILLIAM H. STAHL
Quartermaster Research and Development Center
Natick, Massachusetts

Acknowledgment

The authors wish to thank Mr. M. L. Bazinet, Quartermaster Research and Development Center, for advice and assistance in obtaining and interpreting the mass spectra data.

LITERATURE CITED

1. GILMAN, H. *Organic Chemistry*. Vol I, 2nd ed., 1943. John Wiley and Sons, Inc., New York, N. Y.
2. KEENEY, M., AND DAY, E. A. Probable role of the Strecker Degradation of amino acids in development of cheese flavor. *J. Dairy Sci.*, **40**, 874 (1957).
3. KEENEY, M. Regeneration of carbonyls from 2,4-dinitrophenylhydrazones with levulinic acid. *Anal. Chem.*, **29**, 1489 (1957).
4. MCELVAIN, S. M. *The Characterization of Organic Compounds*. 1947. The Macmillan Company, New York, N. Y.
5. PATTON, S. The mechanism of sunlight flavor formation in milk with special reference to methionine and riboflavin., *J. Dairy Sci.*, **37**, 446 (1954).
6. PATTON, S. The broth like flavor of methional. *Food Technol.*, **10**, 10 (1956).
7. PATTON, S., AND JOSEPHSON, D. V. A method for determining significance of volatile flavor compounds in food. *Food Research*, **22**, 316 (1957).
8. PIERSON, E., GIELLA, M., AND TISHLER, M. Synthesis of DL-methionine. *J. Am. Chem. Soc.*, **70**, 1450 (1948).
9. WITTING, L. A., AND BATZER, O. L. Relation of methional to the odor of irradiated meat. *Food Research*, **22**, 237 (1957).

Errata: In the paper, "The Responses of Hamsters to a Natural-Type Diet containing Emulsifiers," FOOD RESEARCH, 22, 273–286 (1957):
Figure 1 (page 276) change "rats" in legend to "hamsters"
Figure 2 (page 280) change "rats" in legend to "hamsters"

INSTITUTE OF FOOD TECHNOLOGISTS
SCIENTIA ET MACHINA